

Revolution, but without change

The British government is again brooding about its mechanism for supporting basic science. The best solution is radical change without the appearance thereof. The danger is the inverse.

THE British government and its science establishment do not have long to decide what will be the future pattern of research support. By the end of this year, the government needs to be able to say what arrangements there will be for the financial year beginning fifteen months later; will there be five autonomous research councils, as at present, or only one? The question need never have arisen, and did so only accidentally; an urbane but otherwise impatient industrialist, Mr J. R. S. Morris, was appointed a year ago to adjudicate on a turf dispute between the research councils responsible for basic science and medical research (the disputed ground was biotechnology), but the terms of reference were so appropriately genteel that Morris (he had four colleagues as well) was virtually free to advise on what he chose.

It is no surprise that Morris discovered that science is a seamless web, that all partitions of science are bound to be illogical and that it would be better to lump everything together under one larger research council with a universal remit to support research. But the Morris report is more cogent than that. These days, there is a surprising concentration of research at the boundaries between the interests of the five research councils. This is not mere accident, the way an ancient cookie crumbles, but a sign that, beneath often forbidding exteriors, all the research councils compete more vigorously with others than with themselves. Operationally, an interesting interdisciplinary research proposal is bound to seem more appealing than another written straight down the middle of a well-trodden road. The case for returning to a less adventitious pattern is strong, but not sufficient in itself.

The most conspicuous danger in a further reorganization of the means of supporting basic research in Britain is typified by the results of the endless fine tuning of the past quarter of a century. Every few years, some committee or another reaches the conclusion that logic requires a slightly different way of doing things, some energetic minister listens and then effects a change approximating the committee's recommendations — and a few more handfuls of creative people are prematurely put out to grass or, worse, required by their terms of appointment to work on problems that do not interest them. Surely it must now be plain that the scientific community in Britain has had as much of that as it can stomach. The Morris recipe should stand or fall by whatever extra it can offer.

The most important need in Britain now is that a few

handful of very young and very able people whose ability is widely recognized should be able quickly to accumulate the resources (cash and people) required for outstanding original work. Putting all five research councils in the same building and giving them the same letterhead will not make such a state of grace more probable. Sadly, for tidy administrators, there is no single formula under which grants for people such as these can be dispensed. But there also needs to be a measure of continuity: no purpose would be served by giving the whole research enterprise the impression that it has served its purpose. That argues for a weasel's answer to the Morris question; give all the research councils the same letterhead, but take more than 10 per cent and less than 25 per cent of their money away, to be spent quite differently. And, given the fragile condition of morale, the simplest way of effecting that is to pretend that there is to be no reorganization at all, but that the supervisory body called the Advisory Board for the Research Councils will be given executive powers (and the right to spend money) as well as the responsibility for designing the letterhead. □

Nuclear cooking-pot

Unless a major power (or several) intervenes, the Middle East will be a nuclear-weapons playground in ten years.

WHAT can there be in common between the discovery that a branch of the Italian Banca Nazionale del Lavoro (BNL) in Atlanta, Georgia, has been issuing unauthorized letters of credit to manufacturers in the West and a TASS report that a long-range missile was fired last Thursday from a point near Jerusalem into the sea off the coast of Libya? To be truthful, nobody can tell for sure. But if the events, either together or separately, sustain some of the interpretations that are being put upon them, not merely those who live in the Middle East but the rest of us too will be much worse off.

BNL's Atlanta branch is not there to compete with indigenous banks for people's checking accounts, but to lubricate the wheels of international commerce. Letters of credit are one way of doing that; people signing contracts to buy something, but wishing to defer payment, will ask their bank to vouch that the funds will eventually be paid, whereupon the seller can often raise the money



right away. The trouble, at Atlanta, is that an employee of the bank issued letters of credit amounting to more than \$2,000 million to agencies of the government of Iraq without his employers knowing. Iraq is of course the paper victor in the long Gulf War with Iran, and has to replenish its military equipment; the names of the companies in whose favour the letters of credit have been drawn are suggestive of that. But Iraq has also plans to build a long-range missile, and may be buying (or hoping to buy) the necessary components.

So too, of course, does Israel. That the most embattled country in the Middle East should be equipped to defend itself against strategic attack is legitimate enough. But at 1,500 kilometres — the range at which both Iraq and Israel strive — missiles do not make sense as tactical battlefield weapons, because they are insufficiently accurate. Prudent generals will therefore insist that, with missiles of such a range, only nuclear warheads can make sense. They will do so in the process of convincing themselves that deterrence is a way of preventing wars and not of winning battles.

That is where, for the rest of us, the bad news appears. Even generals have to make housekeeping calculations. Would it be better to spend the money on, say, Kalashnikov automatic rifles, or on the means of building and launching 1,500-kilometre missiles and the nuclear warheads to go with them? Hitherto, most generals have acted conservatively, although it is so probable as nearly to be certain that Israel already has a substantial stock of nuclear warheads. Iraq, by contrast, is a long-standing member of the Nuclear Non-Proliferation Treaty (and thus is still rightly indignant that its research reactor should have been illegally attacked by Israel in 1982). Now, with the help of a paper-pusher in Atlanta, it has created the illusion of being a big player. But what if the illusion conceals an identical reality? *Ceteris paribus*, as the economists say, there will be at least two overt nuclear powers in the Middle East ten years from now.

That should keep most of us from our beds at night. A nuclear Middle East would be the Lebanon, as now, writ large. Nobody, especially not those most concerned, would benefit. But how to avoid the prospect? It would help if there could be a conference on the Middle East, but Mr George Bush seems to prefer staying at home and Mr Mikhail Gorbachev has little choice, so that the mayhem in the occupied territories will continue. The International Atomic Energy Agency, run by a Board of Governors representing the world establishment (which meets next week) might exert its influence, but will not. The best way of avoiding real trouble would be for India and Pakistan — whose present leaders are well-disposed towards each other and the future except when one or the other faces trouble (an election or just fractious opposition) — should make a nuclear-weapons deal. Otherwise, there is a danger that the long-withheld promise of a comprehensive test-ban treaty between the open nuclear powers, the best way forward at Geneva now, will simply be overtaken by events. □

Tunnel to nowhere

The British government's neglect of transport's to Euro-tunnel may be a deliberate ploy.

ONCE upon a time, there were inveterate enemies separated from each other by a narrow strip of water called the English Channel, who liked to fight each other at every opportunity, at places with mellifluous names such as Agincourt (the 'g' is soft, as in the middle consonant of 'Brezhnev'). But after many battles and with the passage of time, even these considerable warriors became sated with their victories. (Both had a flair for not remembering, or even recording, their defeats.) So one of them went to Rome to sign a treaty to engage in equal and free trade with its neighbours while the other declined the invitation; everybody sighed with relief, believing that their quarrelsomeness would for ever after be directed against quite different enemies.

Sadly, the outcome has been otherwise, but for a curious reason: most people know geography, but hardly anybody understands it. Living on an island is a splendid state of grace if you believe that everybody else would also like to live there — and if you know they cannot make the journey. But if you have reason to believe that other people do not share your fondness for island life, you quickly discover that island life is a recipe for isolation. The fairy story accounts for British vacillations about the European Community over the past forty years.

More recently, there has been an even more curious happening. The British and French governments, each with ten centuries of recorded history at its back, have come to recognize that people could (and did) cross the English Channel on foot not much further back in time (say, 10,000 years ago). Mindful (as UN communiqués say) that the English (as they were then called) were seized by the fear, at the height of the Napoleonic Wars, that the French might dig a tunnel and walk beneath the Channel, they agreed to exorcize the nightmare by building a tunnel in the full glare of publicity; they would send television cameramen down the tunnel at every opportunity, and would not pay for it themselves, giving its operators a chance to make money from it instead.

Only then did an odd thing come to light: the English, having agreed that there should be a tunnel free for all to use, neglected to make arrangements for conveying people or their possessions from the end of the tunnel to whichever places they wished to go. The British government has agreed to convert about 20 kilometres of country lane near Ashford, Kent, into a modern highway, but has otherwise said that people whose ambitions extend beyond the end of the tunnel must either create their own means of travel or wait until 1995, when there may be a fast railway link if only private entrepreneurs will build it (which is unlikely). It is Agincourt all over again. People from France will be exceedingly irritated, but nothing much in England will have changed. □

NIH restricts university links with industry

- Ban on stock owning
- Too easy to waive rules?

Washington

UNDER pressure either to deal sternly with conflicts of interest in research or to have legislation to that effect imposed on it by Congress, the National Institutes of Health (NIH) last week proposed new guidelines for the conduct of research supported by both private companies and federal funds. Setting the scene for a heated debate ahead, researchers quickly responded that the proposals are too rigid, while Representative Ted Weiss (Democrat, NY) said they give universities too much freedom.

The NIH guidelines, drawn up with the Alcohol, Drug Abuse and Mental Health Administration, impose three prohibitions on institutions receiving NIH funds. No one in a key research or management role may hold equity or options in "any company that would be affected by the outcome of the research or that produces a product or equipment being evaluated in the research project". Neither researchers nor any other "key employee" may receive honoraria, fees or a management position from a private company if they are involved in a federally-funded project that is evaluating the company's product. And no information can be shared with "any company with which a conflict exists" until the information is made publicly available.

The guidelines also stipulate that when researchers receive both private and federal funds, all private support, including instrumentation, consultancies and honoraria, must be disclosed in applications to the NIH and to the institution, and any potential conflict of interest must be resolved before an award is accepted. They also require the institution to ensure that "private companies are not in a position to influence the research plan, results or the reporting or interpretation of results".

Grants awarded through the small business innovation research (SBIR) programme will be exempt from the guidelines the programme was mandated by Congress specifically to increase federal research funds for small businesses.

Weiss, chairman of the House subcommittee on human resources and intergovernmental relations, welcomed the "strong minimum standards", but criticized the "numerous vague waiver and exception provisions which are left to the discretion of the universities". Institutions may grant waivers to the proposed rules if, for example, they decide that the equity holdings are so insignificant that they could not influence research results or the

direction of research. NIH leaves institutions to decide what amount of equity might be considered insignificant.

But Weiss maintains that restrictions should be applied equally. "To allow universities the freedom to determine when stock ownership or honoraria present conflicts of interest would be untenable," he said.

NIH recommends that panels set up to review disclosures and waivers should include one member from outside the institution. All waivers must be reported to NIH, which may allow a conflict of interest to persist if it is in the best interests of the public and of the NIH. But these provisos do not reassure Weiss, who says that "many universities are blind to potential conflicts of interest among their own faculty and will probably rely heavily on exemption provisions".

Universities are unlikely to welcome the proposals. At a two-day symposium on conflict of interest hosted by NIH in June, it became clear that they would resent any restrictions imposed arbitrarily from outside (see *Nature* 340, 7; 6 July 1989). Belle Cole, director of research and public policy at the University of California at Berkeley, criticized the "inflexibility" of the proposals and said that by specifically prohibiting certain situations NIH had "gone further than may have been called for". She said that the onerous reporting requirements of the guidelines would create "a lot of bureaucratic activity".

The chairman of research at the Cleveland Clinic Foundation, Bernadine Healy, said that the proposals were "too vague" and subject to different interpretations. She also said that there would be "a lot of problems" with the prohibition of the release of information to companies before it is made public, as this is usually one of the terms of contracts with industry. And the exclusion of SBIR grant recipients from the guidelines was, she added, "inappropriate . . . and inconsistent with the rest of the document".

But Thomas Malone of the American Association of Medical Colleges (AAMC) said he was "pleased with the approach" NIH has taken. The AAMC and the American Association of Universities are both developing conflict of interest guidelines, said Malone. When these are in place he said, "I'm fairly confident that in the end we'll have a pretty effective system . . . that would offset the need for any legislation".

Christine McGourty

INDIAN AIDS

Penalties — and help — for victims

New Delhi

LEGISLATION introduced in the Indian parliament last week makes AIDS a notifiable disease, and requires regional health authorities to coordinate counselling as well as treatment for people infected by the AIDS virus. But the proposed law also imposes criminal penalties on any HIV-infected person who knowingly donates blood, semen or organs, and gives the government power to quarantine those with AIDS.

Whether these more stringent actions will be put into practice remains in doubt for reasons of both cost and logistics. Each Indian state will be required to designate a health authority to which general practitioners must report cases not only of AIDS but also of drug addiction. The authority is expected to provide health education, counselling, medical treatment and follow-up for notified HIV-positive cases; drug addicts will also be tested for HIV infection. Also introduced will be mandatory HIV-testing for blood donors.

The state authorities are also empowered to remove any infected person to a hospital or to some other place to prevent that person from becoming a risk to society. The bill provides \$10 million for creation of counselling, hospital and rehabilitation facilities.

The criminal penalties in the bill lack one feature recommended by the Indian Council of Medical Research, whose director-general, Dr. A. S. Paintal, had been pushing for more stringent legislation banning sex between Indian residents and foreigners. It is Paintal's belief that most cases of AIDS in India have been, and still are being, brought in by foreigners.

Even before the law comes into force, there are doubts about how effectively it can be implemented. If followed strictly, the new law would require the 1,450 people, mostly prostitutes, known to have AIDS to be rounded up and sent to places of special care or to counselling centres. But no such centres exist.

It is feared that compulsory notification will also drive people away from medical practitioners, who are themselves averse to breaking doctor-patient confidentiality. One physician at a government hospital in New Delhi, for example, has three HIV-positive patients whose wives he has so far not informed; the new law would require him to make the patients' names public.

And no private hospital in India is prepared to handle a patient infected by HIV. A hospital in Bombay that was regularly giving blood transfusion to a thalassemia patient refused to handle her the moment she tested positive for HIV. She got the infection from earlier transfusions in the same hospital.

K.S. Jayaraman

■ AIDS diagnosis — see page 178.

Exxon cleans up, clears off

San Francisco

AFTER "one of the most massive mobilizations of manpower and equipment in peacetime history", Exxon last week officially closed oil-spill clean-up operations in Alaska for the winter. But it leaves behind hundreds of miles of contaminated shoreline, and strained relations with government officials and private parties.

Many officials involved in the clean-up charged Exxon with shying away from an obligation to continue fighting the spill, and Alaska's Governor Steve Cowper announced that the state would continue the work that Exxon has now abandoned, and would submit at least part of the expected \$21 million bill to Exxon.

The 11 million barrels of crude oil that spilled into Prince William Sound last March, when the Exxon tanker *Valdez* struck a reef, has since drifted southwest into the Gulf of Alaska, tarnishing an estimated 1,100 miles of shoreline. The US Fish and Wildlife Service (USFWS) reported last week that 34,434 birds and 994 sea otters had died as a result of the catastrophe, and officials estimated the toll could reach 10 times these numbers.

Exxon has already spent \$1,000 million on the clean-up, including approximately \$90 million to settle claims against itself. The company still faces another 140 lawsuits, including one from the state of Alaska, which is seeking unspecified punitive and compensatory damages for a series of alleged violations of state, common and general maritime laws.

Years of comprehensive scientific studies will be needed to assess how marine life and natural resources have been affected. One early result points to a dramatic decline in bald eagle productivity. In the oiled area, 16 eaglets were born for every 100 nests, compared with 49 eaglets in the Copper River Delta just east of the sound. And two-thirds of the occupied nests in the contamination zone failed to produce any young at all, compared to 29 per cent in the non-oiled area.

To counteract public outcry against its decision, Exxon has stressed that it is not going away. A winter crew of 300 people will be retained, and the company has promised to return in the spring to see what, if anything, remains to be done.

Exxon objects to the accusations that it has not pulled its weight. Otto Harrison, general manager of *Valdez* operations for Exxon USA, says his firm has met its objectives to protect fish hatcheries, skim oil off the water, and treat the affected shoreline by mid-September. The company has 'treated' — the formal term for completing basic clean-up on a stretch of shoreline and receiving permission from



Another environmentally sensitive area was threatened by an oil spill at the weekend — this time the Humber estuary and Spurn peninsula on England's north-east coast. Both are 'Sites of Special Scientific Interest', with large bird populations. The *Phillips Oklahoma* (above) and *Fiona* collided and both caught fire. (Press Association.)

the US Coast Guard to move on — more than 1,100 miles of shoreline. The treated shoreline is, said Harrison, "environmentally stable", meaning that the beaches are in a condition that will not harm marine or animal life.

But state officials are not satisfied. "The term 'environmentally stable' was created by Exxon, really out of the need to say that they had accomplished something", said Joe Ferguson of the Alaska Department of Environmental Conservation. "It is not a term that we use or agree with."

Removing all the oil is impossible, because some has soaked several feet into the shoreline soil. But state officials charge that so far Exxon has provided mainly cursory treatment — such as blasting oil off the tops of rocks with hot water — and that a much more comprehensive effort will be needed next spring.

USFWS is unhappy with efforts to gauge the spill's effect on fall migration of a variety of bird species, including petrels and shearwaters. The USFWS has asked Exxon to maintain at least six boats in the Kodiak Island area to monitor the migration, but spokesman Bruce Batten said that Exxon has apparently decided "to pull out all of the boats. And so we're left with a job that we're still trying to get done with our own resources, and to some extent volunteers."

A kinder judgement came from the Coast Guard, which co-ordinated the clean-up. Exxon has "done as much as could be done in a short period under very difficult conditions", said Vice-Admiral Clyde Robbins, the highest-ranking federal official in Valdez, and added that the oil posed "no great threat" over the winter.

Robert Buderl

HUMAN GENOME

Sequencing bargain in India?

New Delhi

PRIME Minister Rajiv Gandhi is being urged to launch India's own project to map the human genome, on the grounds that other countries which have already begun the task may not share their results with the rest of the world, and that in any case India might be able to do the job more cheaply.

Genome mapping is not a high priority at the Indian Department of Biotechnology (DBT), whose annual budget is \$30 million. But Pushpa Bhargava, director of the Centre for Cellular and Molecular Biology in Hyderabad and a member of DBT's scientific advisory committee, claims that India has "all the capabilities" to do the work, and at a fraction of what the United States plans to spend.

Because most of the money spent on the project would go towards salaries, India could map the entire human genome for less than \$200 million spread over 15 years,

according to Bhargava. He also argues that India could put itself in a good bargaining position with other countries who want the results. But a more compelling reason, Bhargava suggested to Gandhi, is that India might not benefit from human genome work going on elsewhere.

Bhargava's proposal has few supporters, however, and many disagree with his cost estimate. According to S. Ramachandran, secretary to DBT, the project would require not just manpower, of which India has a plentiful supply, but also equipment, reagents and enzymes, which would have to be imported. And G. Padmanbhan of the Indian Institute of Science, one of six laboratories in the country with the facilities to sequence DNA, "would rather sequence the DNA of a pathogenic organism that causes disease in Indians than that of a human being".

K.S. Jayaraman

Making researchers manage

London

THE management of and financial arrangements for British science are outdated and in need of radical change, a former research council head declared last week. At last week's British Association meeting at Sheffield, Sir Douglas Hague, former chairman of the Economic and Social Research Council, told off researchers for being "arrogant", saying that they should be more accountable for the money they spend on basic research.

Hague, chairman of Metapragm Ltd, said scientists could manage science provided they acquired an understanding of economics and management, and the ability to work together in "interdisciplinary teams which include and value those who do have such skills". But he complained that scientists would resist change because they had become accustomed to a long period of increasing expenditure on science.

"Scientists are being selfish with society's money", he claimed, declaring that society had the right "to impose on scientists the responsibility of being accountable for their expenditures and actions".

He described the Advisory Board for the Research Councils (ABRC), of which he was a member for four years, as a "quixotic body" insufficiently experienced in making management decisions. The "amateurism with which it too often operated, when concerned with management and organization", worried him: he singled out as an example the "British Empire Syndrome" — whenever a new research programme was started abroad, the ABRC wanted to carry it out in Britain.

One consequence, Hague said, is that Britain has about half as many research centres as the United States, but "given our relative populations and prosperity, one would expect Britain to have one fifth or fewer of the number in the United States". He also described the civil service as "the joker in the pack", claiming that civil servants are at least as "ignorant as scientists" in making management decisions.

The attack on science management in Britain was sustained by Lord Dainton, the chemist who in his time has been a university vice-chancellor (Nottingham) and chairman of the University Grants Committee. He said it was a "simplistic notion that generous government support for basic and strategic science automatically brings national prosperity".

Because Britain does not have the financial capacity to accommodate the innate tendency of science to expand, he advocated unification of the five research councils to ensure the best deployment of scarce resources, adding that "new opportunities and problems often arise which

can only be tackled by multidisciplinary approaches". The percentage of income spent on bureaucracy would also fall.

The proposal that all the research councils should be amalgamated, put forward earlier this year in a report to the ABRC, will probably be decided in the next few months.

Lord Dainton also concluded that government should divest itself of its existing support for industrial research and use the resources to increase support for universities and teaching. The role of government was to facilitate the movement of young people into industry, because market forces alone would not solve the problem. To get to grips with growing competition for limited funds, Hague argued that researchers should measure the cost of undertaking research not in monetary terms but in relation to the research opportunities which must be foregone, terms in which they normally do not think.

Ben Webb

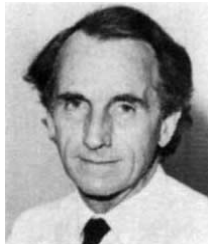
CANCER RESEARCH

CRC and MRC to shuffle the pack

London

THE Institute of Cancer Research (ICR), the largest comprehensive cancer centre in Britain, has created a new post of chief executive in a radical change in management structure. Biochemist Peter Garland will join ICR on 1 November to run a restructured management team.

The new appointment follows the resignation of ICR Director Robin Weiss, who had acted as both director and chief executive while remaining



active in research. ICR Secretary Jonathan Kipling said the Committee of Management decided to take the opportunity to review the management structure.

Garland: to run the The ICR now re-institute in line with ceives £5 million a CRC national strategy. year from both the Cancer Research Campaign, one of Britain's largest cancer charities, and the Medical Research Council, but MRC is phasing out its contribution over the next eight years and CRC will shoulder the lion's share of the budget.

Garland, who leaves Amersham International after two years as director of research, said he was delighted at the prospect of leading the ICR from the foundations laid by Weiss. Amersham has had to cut back on its research programmes after a fall of profits in the last financial year.

Ben Webb

Smithsonian's new formula

Washington

DECLARING that the "anguish" felt by Native Americans was "a weight that one ultimately cannot carry", Robert McCormick Adams, Secretary of the Smithsonian Institution, announced last week an enlarged policy for the return of skeletal remains to Native American tribes where there is a "preponderance of evidence" connecting the bones with modern descendants.

The agreement between the Smithsonian Institution, one of the largest holders of Native American remains, and a variety of American Indian groups comes shortly after a decision by Stanford University to return its archaeological collections to local tribes (*Nature* 340, 9; 1989), and is an example of an increasing recognition by museum curators that the demands of scientific research do not outweigh the proper and deeply felt religious feelings of modern American Indians.

Discussions aimed at loosening the Smithsonian's earlier policy, under which bones were to be returned only if a direct genealogical link with living descendants could be demonstrated, have been going on for many years. But resolution of the dispute was hastened at least in part by the Smithsonian's desire to pass through Congress legislation creating a National Museum of the American Indian in central Washington. With an amendment incorporating the new agreement, such legislation was approved last week by a House of Representatives subcommittee on libraries and memorials; Smithsonian officials feel confident that the new museum will eventually gain the full approval of the Congress.

But a more important element in reaching the new agreement was, according to a spokesperson for the Smithsonian, the growing conviction of Secretary Adams that the Native Americans' grievance is profound. Adams now has the task of appointing a five-member committee, of whom three will be Native Americans, which will judge demands for the return of remains.

In his announcement of the new policy, Adams estimated that 5–10 per cent of the Smithsonian's collection might ultimately be returned for reburial, but wording of the agreement leaves it entirely up to the committee to decide what is or is not a "preponderance of evidence" in favour of returning bones. Some American Indians want all remains, even those thousands of years old, taken from museums and put back whence they came, but curators at the Smithsonian believe this is a minority view, and that most of the collection will be left intact.

David Lindley

How green is Japan

Tokyo

AT A conference in Tokyo last week, the heads of the World Bank, the United Nations Environment Programme (UNEP) and the World Meteorological Organization (WMO) lavished praise on Japan for its contributions to the protection of the global environment. But in a series of independent meetings in Tokyo and elsewhere, representatives of non-governmental organizations (NGOs) from all over the world severely criticized Japan for assisting the destruction of the environment in developing nations.

The conference on the Global Environment and Human Response toward Sustainable Development, sponsored by the Japanese government and UNEP, was proposed last year by then Prime Minister Noboru Takeshita. But unlike a similar event organized by UK Prime Minister Margaret Thatcher earlier this year, the Tokyo conference excluded the public as well as NGOs, some of which then organized rival meetings in Tokyo, Kyoto and Osaka.

The government-sponsored conference was the forum for a string of laudatory addresses. Barber B. Conable, president of the World Bank, praised Japan's endorsement of anti-pollution policies, its development of energy-efficient technology and its decision to emphasize environmental activities in its international development assistance programmes as "salutary examples" of how an industrial nation can adapt its policies to meet environmental challenge.

In the same vein, Godwin Oli Patrick Obasi, Secretary-General of the WMO, expressed the world community's "debt of gratitude" to the Japanese government for its agreement, three days earlier, to establish the WMO World Data Centre for Greenhouse Gases in Tokyo. And Mostafa Tolba, Executive Director of UNEP, praised Japan for its "visionary and generous leadership" in providing \$2.3 thousand million in Overseas Development Aid (ODA) for protection of the environment in developing countries.

Prime Minister Toshiki Kaifu mentioned Japan's intention to "increase" environmental overseas aid to approximately \$2.25 thousand million (¥300,000 million) for the next three years, a commitment made by former Prime Minister Sousuke Uno at the Paris summit in July.

But this figure does not in fact represent an increase in spending. In 1988, environmental ODA commitments on a bilateral basis totalled about ¥110,000 million. And the consequences of Japanese ODA formed one of the main targets of criticism by environmentalists at the NGO conferences.

Martin Kohr from Friends of the Earth

in Malaysia, a speaker at an International People's Forum in Tokyo, said that Japanese ODA has been used to finance the building of logging roads in Sarawak where tropical rain forests are being rapidly depleted by Japanese-backed logging activities. And many other participants from South-East Asia complained that Japanese aid is often tied to Japanese commercial interests, and is environmentally destructive.

At the same conference, Bruce Rich of the Washington-based Environmental Defense Fund criticized Japan's representative at the World Bank for failing to speak out on environmental issues. Although the World Bank has in recent years taken a more active concern for the environment, said Rich, Japan's director of the bank has been "noticeable by his silence" on controversial projects named by the World Bank such as the power sector loan for hydroelectric dams in the Amazon in Brazil and the Sardor Sarovar Dam in Western India.

Rich, Kohr and several other environmentalists, including an Amazonian Indian, Paulino Paiakan, chief of the Kayapo Indians, were able to express their criticisms of Japan in a two-hour meeting with members of the Diet.

But several Diet members left halfway through the meeting and those who

remained were given only five minutes to respond to the criticisms. Eimatsu Takuwa, member of the House of Councillors, complained that he has been trying without success to persuade the Diet to introduce a law requiring environmental impact assessment of ODA projects.

Japan, the world's largest importer of tropical timber, seems unlikely to change its policy towards Sarawak. Taizo Watanabe, a spokesman for the Foreign Ministry, was reported in Japanese newspapers last week as saying that Japan has no intention of reducing or limiting imports of tropical wood.

Watanabe cited a UNEP report which says that Japan's imports account for only 2 per cent of the wood cut in Asia. But the figure is misleading: quoting the same report, Japan's Environment Agency says in its 1988 White Paper that, while migrating landless farmers and agricultural cultivation account for most of the tropical rain-forest destruction in Asia as a whole, in insular South-East Asia (Malaysia, Indonesia, Philippines and Papua New Guinea), commercial logging is "the chief culprit of degradation and indirect cause of deforestation".

In Sarawak alone, according to Kohr, commercial logging destroys about 300,000 hectares of primary forest each year while slash-and-burn for agriculture results in a loss of only 9,000 to 16,000 hectares. Nearly all of Sarawak's log exports go to Japan. **David Swinbanks**

WORLD HEALTH

Health for all by year 2000?

Paris

REPRESENTATIVES of the 32 member states of the European region of the World Health Organisation (WHO) met in Paris last week to reconsider how to achieve their common aim of "health for all by the year 2000". Five years into the programme, member states have another ten years to achieve their 38 goals, ranging from adequate housing and clean water to health provision and research.

The European programme follows a 1977 WHO decision to orient its policies towards health rather than illness. The idea of health for all by 2000 "is not just a slogan" says a WHO communique. Sixty-five health indicators have been agreed by member states to allow quantitative monitoring and comparisons within Europe every four years. "This", says Jo Asvall, director of the European region of WHO, "changes the reality of health".

The idea of health for all was agreed in 1978 and the 38-point strategy was adopted in 1984. By the year 2000, WHO wants to see mean life-expectancy at birth increase by ten per cent to 75 years, to reduce infant mortality, and to eliminate measles, poliomyelitis, newborn tetanus,

diphtheria, congenital syphilis and indigineous malaria.

Other aims are to reduce the frequency of deaths from road accidents, heart disease, cancers and suicides, especially among the young. Some of the goals are closer at hand. By 1990, WHO wants Europe to have "sufficient supplies" of drinking water and, by 1995, to have made "considerable progress" in the campaign against smoking.

But a new report* on the state of health in France suggests that this latter goal may be hard to achieve. Although mean life expectancy in France is now increasing by four months every year, the number of deaths from smoking-related illnesses is increasing and an estimated one in five males smoke more than 20 cigarettes a day.

The increase in life expectancy means that the proportion of the elderly in the population is increasing. In France, there will be an estimated 1.2 million elderly (over 85 years) by 2000. **Peter Coles**

* *La santé en France — faits majeurs et grandes tendances*. Published by La Documentation française, September 1989.

LARGE TELESCOPES

Six feet on the ground

Munich

THE University of Bochum has broken new ground with its entry in the competitions for the funds the federal government has set aside for building a new large telescope. But Bochum's daring has attracted critics as well as admirers.

Bochum is hoping to be the beneficiary of an anticipated DM 200 million (\$102 million) the West German government is thinking of spending on a new German Large Telescope (GLT). Theodor Schmidt-Kaler and his colleagues at the university last month revealed a revolutionary "hexapod" design for a large optical telescope. Like all modern telescope designers, Schmidt-Kaler wants to keep both weight and costs down without losing optical quality, but plans to achieve this by building an instrument steered by six legs, all moving independently.

But Klaus Fricke of Göttingen University, leader of a rival group with a conventional design, says "half the world is laughing" at Schmidt-Kaler's proposal, and hopes his group will win the project.

The design for the Bochum hexapod telescope (HPT) was inspired by airline flight simulators, whose position is controlled by piston-like legs. The HPT would have the advantage, according to Schmidt-Kaler, of allowing equally accurate pointing of the telescope across the whole sky. Conventional altitude-azimuth mountings usually have a blind-spot overhead.

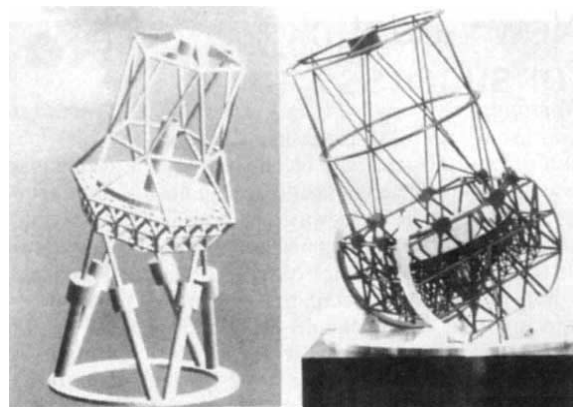
The second advantage of the HPT is its relatively low weight. Schmidt-Kaler and his colleagues, Gerhard Schur of Bochum and Karl-Heinz Stenvers of Krupp AG, have had been experimenting with lightweight carbon fibres, and claim that if the HPT mounting is made of these materials the total weight of a 1.2-metre-diameter telescope (the size of a planned prototype) need be only 2 tonnes, as against about 30 tonnes for a conventional design. For the 12-metre telescope that is ultimately planned, this would yield a weight of 120 tonnes, lighter than much smaller conventional telescopes.

The rival project from Fricke's "GLT working group" will be unveiled in the next few weeks. Zeiss AG is collaborating in the project. The proposal is based on a conventional Cassegrain optical system, but uses a new type of segmented mirror. Unlike the US Keck telescope, whose main mirror consists of 36 similar hexagonal elements, the GLT would have a central segment 8 metres in diameter surrounded by smaller mirrors.

Fricke is "extraordinarily sceptical" of the hexapod design. Schmidt-Kaler, he said, is "isolated from the German astronomy community", in part because he "betrayed our interests" by announcing

the HPT so early and by not informing the GLT working group of his plans. Fricke said there had been a tacit agreement not to talk about GLT for two years after the West German Research Ministry agreed in November 1987 to pay a large share of the costs for the Very Large Telescope of the European Southern Observatory (ESO).

Schmidt-Kaler rejects Fricke's assertions. He said that he kept the GLT group informed at all times of his intentions and that he is "convinced" that the HPT project will lead to a large telescope. As far as the timing of the announcement, Schmidt-Kaler would only say that Krupp had been responsible for that. The telescope race also matches Krupp against Zeiss, two firms known for producing excellent telescope technology.



The hexapod (left) in computer graphic form, and a model of a conventional mounting for the GLT.

An independent observer, Ray Wilson of ESO, called HPT an "exciting idea" and an "admirable project." But he warned that building a successful HPT will be difficult because the length of the legs must be adjusted over a large range with great accuracy. Nevertheless, "if anyone can make this thing work", said Wilson, "the Bochum-Krupp group can".

Steven Dickman

SCIENCE AND CITIZENS

An unobtrusive neighbour

San Francisco

THE vast medical research, teaching and hospital complex of the University of California at San Francisco (UCSF) has received a clean bill of health from a 20-month environmental study. The study, which cost \$1.6 million, is likely to help smooth the rocky relations between the university and citizens' groups whose concern about toxic emissions prompted public disclosure of the medical school's health and safety practices. The campus is in the heart of a residential district near San Francisco's Haight-Ashbury district.

The study may prove a model for universities elsewhere in the United States. UCSF officials say their environmental assessment is the most comprehensive ever undertaken by a medical centre, but, given increased public concern over the environment, other studies are sure to follow. The assessment was carried out by Radian Corporation, an independent company from Sacramento, which examined air, water and soil samples from on or near the campus. It found no detectable effect on air quality and concluded that chemicals in sewer water were a fraction of allowable government levels.

University vice-chancellor Bruce Spaulding says the study shows that medical facilities pose no significant risk to their neighbouring communities — and that the UCSF experience provides a lesson for similar facilities nationwide.

Kathe Traynor, who represents the Haight-Ashbury Improvement Association, says the new study bolsters UCSF's

community relations around the present campus. But a planned move of the School of Pharmacy research laboratories to the Laurel Heights area of San Francisco is still at issue. A lawsuit filed by the Laurel Heights Improvement Association of San Francisco Inc. challenged the plan — and late last year the state supreme court ordered UCSF to undertake a new Environmental Impact Report (EIR) in order for the relocation to be approved (see *Nature* 336, 706; 1988).

Robert Buderl

SITUATION VACANT

Still no taker for NIH directorship

Washington

THE Bush Administration is still casting about for candidates to fill the post of director of the US National Institutes of Health (NIH). The top three candidates on the short list have declined the job.

Anthony Fauci, director of AIDS research at NIH and head of the National Institute of Allergy and Infectious Diseases, removed his name from the list two weeks ago. The drug company Merck has indicated through its press office that its president, P. Roy Vagelos, will not be leaving the company.

William H. Danforth, chancellor of Washington University in St Louis, has also asked not to be considered for the post. James B. Wyngaarden, the previous NIH director, resigned at the end of July.

Carol Ezzell

New yardstick for success

Washington

THE use of mortality rates, or of progression to AIDS, as the only objective way of assessing the effectiveness of drugs against the disease may be on its way out. A public conference last week, sponsored by the US Institute of Medicine, concluded that it may be sufficient proof that a drug is working that there should be evidence that it prevents patients' levels of CD4 lymphocytes from falling, at least for the purpose of telling whether a clinical trial should be ended.

Using such a "surrogate endpoint" should allow promising drugs to be identified more quickly than at present. This could be important because early treatment for HIV infection has recently been shown to postpone the development of symptoms.

Counts of CD4 lymphocytes — white blood cells of the type infected by HIV, the virus causing AIDS — have long been used by people with AIDS as a rough indicator of the progress of their disease. But the counts must be made using laser cell-sorting, a technique whose results differ from one laboratory to another, making the data from various medical centres participating in a clinical trial difficult to compare. Measurements taken by centres conducting trials under the aegis of the US National Institute of Allergy and Infectious Diseases are now being standardized.

CD4 counts also vary broadly in an individual according to the time of day. Ratios of CD4 to CD8 lymphocytes are often a more useful indicator than absolute numbers of CD4 cells. But increases in the amount of CD4 cells are usually short-lived even when a given drug is having a positive effect.

Assays for the p24 core protein of HIV and tests using the polymerase chain reaction to estimate the proportion of infected CD4 cells may be useful in monitoring the status of individual patients, but their use as endpoints in clinical trials is distant. Levels of HIV core protein in the blood rise when the virus is actively reproducing, but the protein can be detected only in roughly two-thirds of those infected with HIV. The polymerase chain reaction is used extensively in research, and it can be used to determine if a baby born to an HIV-infected mother has been infected. But it is sensitive to contaminating DNA from sources other than HIV, and results from different laboratories may vary.

The shortcomings of individual tests may be overcome by using several together. But measurements of CD4 cells may be the first surrogate endpoint to be used as a means of obtaining drug approval: a pediatric clinical trial of AZT that ended two months ago may provide the test case.

Carol Ezzell

No blanket approval

Washington

THE US National Research Council (NRC), sponsored by the three academies of science, engineering and medicine, this week proposed a new framework for assessing the risks involved in releasing genetically engineered organisms into the environment. Although the report rejects the need for new legislation or a tight system of licensing and regulation, it may counteract the effect of a previous NRC report interpreted by some as giving blanket approval to genetic engineering.

The new report was commissioned by the Biotechnology Science Coordinating Committee, which oversees all five of the federal regulatory agencies with responsibilities for monitoring releases.

Its proposals could bring some uniformity to the present "hodgepodge" system, according to Robert Burris, of the University of Wisconsin at Madison, who was chairman of the steering committee responsible.

The NRC recommends an approach similar to that proposed in February by the Ecological Society of America and welcomed by environmental groups. Even so, Margaret Mellon of the National Wildlife Federation (NWF) is disappointed that the NRC did not provide a means for involving the public in decision-making, but Burris replies that "everything is aimed at trying to make the approach rational so that the public won't have to worry about it".

Instead of attempting to categorize in advance different products according to their potential risk, NRC proposes weighing on a case-by-case basis the characteristics of each organism and its potential effects on the environment, with no reference to the means used to modify the organism genetically. Mellon says that, by not expressly excluding any product from scrutiny, NRC is endorsing a broad net of regulation.

Although NWF is calling for a tight system of registration and licensing similar to that proposed in Britain (see *Nature* 340, 84; 1989), NRC says that "the complexity and cost of seeking regulatory approval" has discouraged academic researchers in the United States from carrying out field testing of engineered plants. On the contrary, researchers should be encouraged to use genetic engineering, it says.

Three major criteria, "familiarity, control and effects", are proposed for assessing the risk of release of genetically engineered microorganisms. To satisfy the "familiarity" criterion, the microorganism, its intended function and the target environment must be "sufficiently similar to prior introductions that have a safe history of use". Small-scale field tests can then proceed without the need for any

increased oversight.

Releases that do not satisfy this criterion are to be assigned to low-, moderate- or high-risk categories according to people's ability to control the persistence and dissemination of the microorganism as well as its potential for significant adverse effects.

Burris says that existing institutional review committees could adopt the proposed framework. Local oversight should be enough for low-risk field testing — probably 95 per cent or more of all current research — and federal oversight could be used for "the few cases where perceived risk exists".

Christine McGourty

AUSTRALIAN FAUNA

Koala researcher faces charges

Melbourne

A CONTROVERSIAL Australian koala researcher, widely known for his view that koalas are becoming an endangered species, is now facing criminal charges and a fine of up to \$80,000 as a result of his research activities. After an investigation by police and the Queensland National Parks and Wildlife Service (NPWS), Stephen Brown, director of the Koala Research Centre at Queensland's Bond University, has been charged with three counts of transporting and two counts of keeping protected fauna without the appropriate authority.

Brown claims that koalas are endangered because of reduced fertility in females as a result of widespread infection by the bacteria *Chlamydia psittaci*. But his claims have been criticized by other Australian researchers since the first koala biology symposium in 1976. Despite the release of statements at subsequent symposia saying that koalas did not face extinction, Brown has continued to gain public attention, and his research has attracted large corporate and public donations.

Kath Handasyde, of Monash University in Melbourne, says that although researchers have found that some populations of koalas have indeed suffered lower fertility because of chlamydia, surveys have shown that koalas are not endangered. Ronald Strahan, a former researcher at the Australian Museum and convenor of the koala symposia, says that some other researchers, have disagreed with Brown, but that they have been reluctant to voice their criticisms.

The NPWS has now cancelled Brown's fauna permits, preventing him from carrying out his research. His fauna records have been seized and a spokesman for NPWS says further charges may be brought against him.

Tania Ewing

SOVIET SPACE

Wings clipped by economics

London

THE Soviet space programme will have severely practical objectives in the next 15 years. The guiding principles during that period will be those of what is called Programme 2005, and will be concentrated in three main areas: the modernization of commercial satellites, wider use of space facilities for research and more manned flights in Earth orbit, according to the government newspaper *Pravitsel-tvennyi Vestnik*.

Special emphasis, the newspaper says, will be placed on the satellite monitoring of environmental pollution, satellite broadcasting and satellite emergency rescue systems, as well as map-making and weather forecasting. The manufacture of semiconductors and sophisticated drugs in zero gravity will also have priority status.

During the past 33 years, the paper said, the Soviet Union has spent less than 20,000 million roubles on the development and maintenance of commercial space facilities, while space research has generated some 12,000 million roubles as spin-off. Current annual spending on space is 1,700 million roubles, some "five to ten" times that of the United States, and less than 1 per cent of the All-Union State Budget.

Programme 2005, the report said, will involve the Soviet Ministry of General Machine Building in an outlay of around 6,000 million roubles in 1991-95. This breaks down into 1,000 million roubles for the development and construction of new commercial satellites (excluding their maintenance costs), 2,000 million for research and 3,000 million for manpower costs and new hardware. The newspaper says the programme should produce benefits worth up to 27,000 million roubles — a yield of almost 400 per cent.

The publication of these figures, unprecedented in the history of the Soviet space programme, is not simply an application of *glasnost* to a previously secret area, but is also relevant to recent debate about the cost of the space programme, for many years a subject of dissident black humour now being openly questioned.

A number of the new People's Deputies have used the new parliamentary procedures to express their doubts that space can ever show the cost-effectiveness which is the basis of Mr Mikhail Gorbachev's economic reforms. The correspondence pages of several leading newspapers have carried plaintive and angry letters asking when all the outlay on communications satellites will supply an efficient telephone service.

Soviet journalists, perhaps piqued that a Japanese citizen will be the first pressman to visit the Mir space station, and

only partially mollified by the founding of a journalism contest whose winner will get a similar trip, no longer greet every new launch as an unqualified triumph. Last month, indeed, *Izvestiya* published a full-page exposé of the aborted manned Moon programme of the 1960s, the existence of which was not officially acknowledged during the Brezhnev years.

Then — the unkindest cut of all — Dr Boris Egorov, Director of the Soviet Ministry of Health's Centre for Medical Biotechnology, and the first-ever physician in space, recently took advantage of a *Pravda* interview to "protest categorically" against the assertion that new medicines can be produced in space, thus calling into question one of the kingpins of Programme-2005.

But Soviet space planners are fighting back with all the weapons that *glasnost* allows. Major launches, once a secret until after blast-off, are now announced in advance. The commercial value of missions is stressed — the latest manned launch even carried commercial advertising. The Interkosmos joint Comecon programme is now officially commended as a demonstration of Soviet managerial skills and — perhaps in response to the current upsurge of ethnic awareness — the role of non-Russians in the space programme is receiving special mention, from the Ukrainian head of the Baikonur cosmodrome and his Lithuanian on-site political chief down to the 62 ethnic groups represented among the staff at the base.

Vera Rich

POLISH CABINET

Academics in high office

London

POLAND's new minister at the Office of Scientific and Technical Progress is to be Dr Jan Stanislaw Janowski, currently rector of the Academy of Mining and Metallurgy in Krakow. Dr Janowski is a long-standing member of the Democratic Party (SD), one of the two small parties which, for the past 45 years, have voted together with the ruling Polish United Workers' Party. Since 1976, Dr Janowski has represented the SD in the then single-chamber parliament.

In 1982 he found himself in serious trouble with the SD leadership when he refused to vote the party line supporting the outlawing of Solidarity. He returned to party favour only in 1985, when he became deputy leader of the SD parliamentary group, becoming leader after the "semidemocratic" elections last June.

Last month, when the SD and the equally small Peasant Party (SL) withdrew their support from the Polish United Workers' Party, Janowski was one of the advocates of the coalition which eventually led to the formation of a Solidarity-led government. Janowski will combine ministerial duties with the role of deputy premier.

The education portfolio goes to Dr Henryk Samsonowicz, a historian, who was elected Rector of Warsaw University under the democratic procedures of the first Solidarity period (1980-1981) — and summarily relieved of that office when Martial Law was declared.

Vera Rich

PALAEOPHILATELY

US Post Office flunks fossil test

Washington

THE US Postal Service, financially straitened and recently chided by Consumers' Union for its worsening delivery rate, has now had to admit that its knowledge of dinosaur nomenclature is behind the times. At the urging of Congressman George Brown (Democrat, California) a new set of dinosaur stamps will be issued, on 1 October, with a statement to the effect that what the Postal Service says is a *Brontosaurus* should properly be called an *Apatosaurus*.

The name *Brontosaurus* was given by



Charles Marsh to a fossil creature he discovered in Colorado in 1874, and soon became part of every schoolchild's common knowledge. But in 1974 palaeontologists agreed that Marsh's *Brontosaurus* was identical with a previously discovered dinosaur known as *Apatosaurus*, and that the prior name should take precedence.

The Postal Service had used the old name deliberately, on the grounds that it is more familiar to most of the population. But Brown was concerned that a generation of schoolchildren would be dangerously confused without official clarification.

David Lindley

■ The UK Post Office is being asked — by the Plymouth branch of the British Association — to follow the fashion for dinosaurs on stamps. The occasion is the 150th anniversary, in 1991, of the society's 1841 meeting, when Sir Richard Owen, first director of the Natural History Museum, identified the bones of a large reptile for the first time as *Dinosauria*. □

Legacy of the Nazis

SIR—The recent discovery in German institutions of anatomical specimens derived from victims of the Nazis has profound implications for medicine and medical science. *Nature* is the only scientific journal so far to report these findings. The relative silence of the scientific press appears to support the concern expressed by the director of one of the institutions involved, the Max Planck Institute of Brain Research, "that the reputation of science might suffer from further publicity".

While Western medicine is seeking to come to grips, in a meaningful way, with medical ethics, it overlooks an issue that challenges the ethical basis of medicine. Ironically, modern medical ethics arose from the experience of Nazi medicine, in particular medical experimentation conducted on unwitting subjects imprisoned in concentration or death camps. The Nuremberg code on medical experimentation was formulated at the War Crimes Tribunal; have medical ethicists forgotten the origins of their discipline?

Now, ironically, while responsible German institutions acknowledge the possession of these specimens and are considering plans for their proper disposal, the medical and scientific community elsewhere remains largely ignorant and silent on the issue. In the long term, that will damage the reputation of science and medicine. Although the medical profession has now effectively dissociated itself from Nazi medicine, which is regarded as an isolated example of evil practised by a few SS physicians, in reality Nazi medicine involved virtually the entire German health-care system in racial selection, enforced sterilization, 'euthanasia' of the mentally ill, research into racial hygiene and genetics and various forms of forbidden medical experimentation. Courses on racial hygiene became part of the undergraduate medical curriculum^{2,3}. Yet medicine today continues to ignore the links between medicine in Hitler's Germany and medicine elsewhere.

Scientific medicine developed, for the most part, in the Germany of Koch, Virchow, Roentgen and Erlich. The institutions that acquired and used these specimens are part of the system of medical education that was a model for the modern North American system. Abraham Flexner, the architect of the North American system of medical education, saw the German university system as a model for merging the university and science in the training of physicians⁴. The Nazi physicians responsible for some of the worst evils in recorded history were graduates of this same university system.

That is why this tragic episode of these anatomical specimens must become more

widely known. The specimens should not only receive a respectful burial but their existence and burial should be acknowledged and commemorated by all of medicine. There must be public documentation of who those people once were, how they died and how institutions representing science, medicine and higher education used their remains for almost half a century after the defeat of the Nazi regime. A proper documentation and public burial would help to sensitize the profession, and the world, to the fallibility of medicine and the vulnerability of human society.

WILLIAM E. SEIDELMAN

North Hamilton Community
Health Centre,
554 John Street North,
Hamilton, Ontario L8L 4S1, Canada

1. Dickman, S. *Nature* **337**, 195; **339**, 498 (1989).
2. Müller-Hill, B. *Murderous Science* (Oxford University Press, 1988).
3. Proctor, R. *Racial Hygiene: Medicine Under the Nazis* (Harvard University Press, 1988).
4. Flexner, A. *I Remember* (Simon and Schuster, New York, 1940).

Stanford maligned

SIR—I have just read Alex Comfort's review of *Stalking the academic Communist: Intellectual freedom and the firing of Alex Novikoff* by David Holmes (*Nature* **339**, 347; 1989).

In it, the following sentence appears, in connection with Comfort's claim that academics generally show cowardice in the face of external attack: "If anyone thinks that this was a feature of a unique period, they should have been at Stanford a few years ago when an equally malicious but non-political attack on a distinguished scientific faculty member—also Jewish—found his colleagues, including his Jewish colleagues, looking at their blotting pads in equal silence."

I have been at Stanford for 30 years as a member of the science faculty, and for the past nine as its president. I have no idea what Comfort is talking about. For all I know, there may be a case to be made somewhere. But Comfort doesn't make it; he merely inserts a piece of offhand institutional slander so lacking in documentation that one cannot even identify its source.

DONALD KENNEDY

Stanford University,
Stanford, California 94305-2060, USA

Guidelines needed

SIR—Authors of research papers are apparently not the only ones in the scientific enterprise in need of publication guidelines and oversight mechanisms. A journal editor recently held a manuscript of mine

for four months before it became clear that he had never sent it out for review and did not wish to do so until I could be persuaded to cite his research. When I refused (because his papers were only marginally relevant), he reluctantly proposed to send the manuscript to two reviewers, with, he said, "my anonymous comments regarding its merits and faults".

The behaviour of this editor is, I believe, contrary to custom and certainly to what the scientific community expects. Such lapses could, to some extent, be prevented or redressed. On the page or pages where a journal now gives its instructions to authors (often demanding a lot in the way of form and format), it should also set out its own guidelines on editorial policy, and say who is responsible for them. It could be stated, for example, that the society's board of directors, or the publishers, had agreed on the listed guidelines. These would include how a manuscript is processed, how many reviewers are used, how long a decision is expected to take, and so on. It is important that a publisher or board should participate in setting guidelines and, when necessary, take part in implementing them. Abuses go unreported in systems where the culprit has been appointed judge, jury and court of appeal.

RICHARD F. SHAW

10201 Grosvenor Place, Apt 522,
Bethesda, Maryland 20852, USA

German or Polish?

SIR—Steven Dickman writes (*Nature* **340**, 497; 1989) that the 'Hall of Fame' of the German Museum in Munich "contains busts of 38 'German' scientific greats, from Copernicus (a Pole) to Einstein", thus implying that not all the 'greats' were German, and certainly not Copernicus. One could well argue about the purpose of halls of fame, and Copernicus himself would probably turn in his grave at the thought of Poles and Germans staking claims to his presumed loyalties, but, to be fair to the museum, he was born into an ethnic German family and raised in a German-speaking community. His native town, Thorn (today's Toruń), was first occupied by Poland less than seven years before his birth, and although now an integral part of the Polish nation, its German population was replaced by ethnic Poles only gradually during the past 100 years. By the criteria of his time, Copernicus was a German, as his association with the German 'nation' among his fellow students at Padua University illustrates; by the current definition of nationality, maybe he was a Pole as well. Copernicus, perhaps, is big enough for two halls of fame.

HERMAN REICHENBACH

Paul-Sorge-Strasse 74,
2000 Hamburg 61, FRG

Sexual behaviour unsurveyed

The British government's refusal to countenance a survey of people's sexual behaviour is demoralizing for those concerned but should not deter them from pushing ahead with the project.

WE are all familiar with the idea that people who work on distasteful projects, say the optimum design of dum-dum bullets fired at mammalian torsos, will cast a pall on the most cheerful company if they talk too openly about their work. But not AIDS researchers, surely? Those I remember most vividly are a group of physicians at the San Francisco City Hospital some years ago; mostly in their early thirties, there could have been no reason, except heroism and the hope that their experience might help the treatment of patients still to fall ill, why they should thanklessly have been running a hospital ward for the indigent and terminally sick. Yet they were a brisk and cheerful lot, and their humour was infectious. No doubt it helps if what you are doing commands most people's approval.

Those in Britain who have been designing the temporarily abortive "National Survey of Sexual Attitudes and Lifestyles" are a different case. Those who have been talking on the telephone are fully aware that little has been done in this field since Kinsey in the early 1950s (and even that, they say, was more like a collection of case-studies with numbers than a survey, given the biased character of the sample). But one whom I have never met except by telephone put the personal predicament strikingly one day last week: "She's made us feel ... well you know, ... naughty." "She", of course, is the prime minister, Mrs Margaret Thatcher, who declined to let British government agencies spend money on the project. The peculiarly English word "naughty" is to be understood, on this occasion, as "prurient".

All research requires that a person should invest his or her reputation in that of what emerges, but there are some fields of research in which people are especially vulnerable to external insult. It seems doubly cruel that the same people have to suffer self-inflicted insults, otherwise what the Freudians would call guilt, born of nothing more substantial than one person's disapproval.

Luckily, other researchers seem to have been mostly encouraging. But I did meet one sociologist last week who volunteered that he'd have been exceedingly cautious of a proposal to measure sexual attitudes and lifestyles (or behaviour?), given the pitfalls that abound in trusting what people will tell even professional pollsters. But in the trade, it seems to be acknowledged that the questions that

most vigorously rouse resentment are about personal incomes, not behaviour. Physicians seem particularly supportive of the project; even on such a scale as that proposed — there would have been 20,000 respondents in the full-scale survey — the data may not pin down the parameters that epidemiologists need to calculate the likely spread of AIDS, but might they not say something about papilloma virus and cervical cancer?

The potential benefits of a substantial survey along these lines would be even more direct. From the data gathered in the the pilot surveys in 1987 and 1988 (some of which were presented at a meeting of the Society of Social Medicine at Manchester last week), it seems clear that there is a great deal to be learned about the sexual behaviour of adolescents that would quickly inform social programmes now designed in ignorance to cater for them.

That the median age of women at first heterosexual intercourse has fallen from more than 20 to 16 in two decades is, for example, unsurprising, given anecdotal evidence. But it is surprising that, even among the youngest age-group (16–24), nearly a quarter of first intercourses are innocent of contraception. In contrast with the older groups, condoms appear on more than half of these occasions, which might be good news for a government such as the British which has spent a great deal on advertising their utility in the prophylaxis of AIDS on television in the past few years. If the numbers responding had been greater, the chance of disentangling the several possible causes of what seems to have been a dramatic shift of practice would naturally have been much greater.

At this stage, when the numbers are small, it is understandable that there should be relatively little that bears directly on the epidemiology of AIDS. The most telling information would eventually have come from a sensitive knowledge of the rate at which people acquire new sexual partners; multiplied by the chance that an infected person will infect another, this quantity measures the chance that people will become infected. The figures so far show that the average number of heterosexual partners of men in the course of a lifetime is 11.0 and of women 2.9. But the rate of spread of sexually transmitted AIDS depends not on the average but on the behaviour of the most sexually active people, which argues again

for the weight of numbers.

The need for larger numbers is further emphasized by the need that a survey intended to throw light on the spread of AIDS should include sufficiently large numbers of those in the high-risk groups, homosexual and bisexual men, for example. Only then can a survey of this kind be related to other kinds of studies, perhaps the tracing of infected people's partners. One of the disconcerting features of the British pilot survey is that the proportion of men reporting homosexual partners is too small to be believable; the succeeding feasibility survey, involving the greater use of self-completed questionnaires, seems to have been more successful.

That appears also to have been true of the response rate, the commonly-used indicator of the representativeness of any survey. In the original pilot survey, the response rate was less than a half, but in last year's feasibility study, it had increased to 62 per cent of those still to be found at their random addresses. But 24 per cent refused point-blank to cooperate, which is too great a proportion for comfort. In passing, it would be interesting to see whether responses to a survey carried out in the next few weeks (there is no time, of course) would have been increased or decreased by the government's refusal to allow this study to be sanctioned, but that is another matter.

Meanwhile, there is a broad question affecting the whole scientific community. The British government is within its rights to say no to whatever it likes, and nobody can justly complain that it has interfered with academics' freedom. On one view, the refusal merely reflects the government's calculation of how many voters dislike the whole idea, and who probably predominate among those who decline to be interviewed. The moral is that the disappointed researchers, but especially their colleagues, should carry the argument for greater awareness to the public.

In the long run, that is how communities such as the British will come to recognise that statistical measures of their behaviour is not an invasion of their privacy, but can be a great social benefit. But there is also a short-term benefit to be won. It is natural that people who have worked for two years on a project suddenly halted should be disconsolate, but the government is not the only possible source of funds. This team deserves a fund-raising drive.

John Maddox

DNA forensics and the FBI

Rory Howlett

FORENSIC laboratories have been quick to realize the potential of the new DNA technologies such as 'DNA fingerprinting'¹, and these have already been widely used as evidence in both the American and British courts. But there is growing unease that in the rush to exploit the power of the techniques, standards have been overlooked². The Office of Technology Assessment, a research and analytical agency of the US Congress, is presently considering the uses of DNA tests. Its purpose is to gather technical information on the reliability of the various techniques, assess costs and procedural issues, and outline policy implications. Many of the relevant issues were discussed at a recent meeting in Atlanta*.

DNA forensics is a very young science. Up to 1985, forensic scientists were largely dependent on well defined protein polymorphisms, such as ABO blood groups, serum proteins and red-cell enzyme systems, when trying to link a particular suspect with forensic samples such as blood stains. On the basis of such markers, an exclusion of 99 out of 100 individuals would be exceptional; semen markers were even less informative. With the advent of so-called 'DNA fingerprinting', however, forensic science has moved from exclusion to positive identification; for the first time there is at least the potential to identify the donor of a body fluid or tissue to the exclusion of all other individuals except identical twins.

Unique mutations

The identification of individuals on the basis of their DNA depends largely on the exploitation of restriction-fragment length polymorphisms (RFLPs). These result from mutations that create or eliminate restriction-enzyme recognition sites in DNA so that the same region of the genome is cleaved by restriction enzymes to fragments of different lengths in different individuals. An important class of RFLPs is that associated with so-called human 'minisatellite' DNA¹. Minisatellites are dispersed regions of tandemly repeated sequences detected through a shared core sequence (similar to the generalized recombination signal, χ , of *Escherichia coli*). Many minisatellite loci are highly polymorphic for length alleles because of variations in the number of tandem repeat sequences present between two restriction sites. Such minisatellite 'variable-number tandem repeats' (VNTRs) can be detected using a probe derived from minisatellite sequences from an intron of the human myoglobin gene. This probe, first described

by Jeffreys *et al.*¹, detects many highly variable loci simultaneously and generates individual-specific 'DNA fingerprints' of general use in human genetic analysis.

Despite the widespread use of the original minisatellite probe, not everybody agrees that it is the most appropriate for forensic work. This point was clearly made at the Atlanta meeting by B. Budowle, a forensic scientist with the Federal Bureau of Investigation Academy, Quantico, Virginia. The FBI favours the use of probes that recognize variable-number tandem repeats that occur only at single loci^{3,4} instead of being dispersed. Such single-locus probes are claimed³ to be more sensitive than the original Jeffreys probes because they bind at higher stringency. The probes produce simple patterns which, says Budowle, is an advantage when banding patterns from different specimens have to be compared. Furthermore, the single-locus probes favoured by the FBI lack the extreme cross-reactivity of the Jeffreys probe. Thus, whereas the Jeffreys probes detect hypervariability in organisms as disparate as rice and whales, so far as is known the single-locus probes cross-react only with DNA from higher primates⁵.

It is widely recognized that several criteria must be met before a genetic marker can be safely used for human identification. First, the background frequency of the marker in human populations must be known. Second, the marker must be stable in forensic specimens and artefacts must be accounted for. Third, the methods and reagents must be readily accessible. Finally, the techniques must have appropriate sensitivity, and standards and required controls must be defined. (The need for care is illustrated in Eric Lander's disturbing account² of the case of *United States v. Castro*.) According to Budowle, the FBI has designed protocols to take into account the potential problems associated with forensic use of the new DNA technologies. Training is stringent and FBI research laboratories provide 'blind' samples so that the competence of those actually doing the testing can be monitored.

Several restriction enzymes are suitable for RFLP typing, but the FBI has settled on *HaeIII* (ref. 6). This is a very robust enzyme which gives consistent banding patterns even when digest conditions are adverse. Furthermore, it is insensitive to the methylation state of its substrate — an important point because otherwise there would be a danger of artefactual differences due to tissue-specific patterns of DNA methylation.

Forensic samples taken from the scene

of a crime are often corrupted by chemical or biological contaminants, or have been exposed to potentially harmful environmental factors such as sunlight. For this reason, the FBI has attempted to validate the use of its single-locus probes by comparing the banding patterns generated from contaminated or environmentally exposed samples with controls⁵. According to Budowle, the data from these studies suggest that the analysis of such samples is valid and reliable and that the chance of a false result (either positive or negative) is negligible, if the tests are competently performed. He believes that such studies will allow courts to assess more readily the weight of the data derived from forensic analyses.

Limitations

Because the quantity and quality of DNA in evidence material can be limiting, it seems that the polymerase chain reaction (PCR)⁷, which is capable of greatly amplifying defined DNA sequences, is likely to become an important adjunct to RFLP typing⁸. Indeed, it has already been shown that PCR can be used to make possible the typing of DNA from single hairs⁹. The FBI is also considering exploiting sequence variability in human mitochondrial DNA for forensic work¹⁰. DNA sequencing is another possibility (G. Sensabaugh, Univ. California, Berkeley), but in this case there will be a need for readily available sequencing technologies with low reading error rates and sequence-variable regions will have to be targeted.

Whatever approaches are deemed most suitable for DNA forensics, it is clear that appropriate statistical methods will have to be developed. Because of the limited resolution of present RFLP technologies, it is often difficult or impossible to distinguish alleles of similar length. To take such limitations of the data into account, Budowle favours the statistical classification of the data into arbitrary fixed 'bins', the boundaries of which are defined by standard markers of known length³. The distribution of alleles falling into different bins for any given population can then be used for all subsequent statistical analyses. Such an approach, Budowle argues, is cautious and conservative in that it will tend to overestimate the frequency of rare alleles in the population; it is, of course, the rare alleles that are most subject to genetic drift, founder effects and sampling error. Because it is also these alleles that tend to give rise to the lowest probabilities of a false match, a cautious approach is necessary, says Budowle, particularly given the sparsity of knowledge about the true background frequencies of VNTR alleles in human populations. In any case, Budowle believes that the main emphasis of forensic work should be to exclude individuals who have been falsely associated with a

*Genetics Society of America, 58th annual meeting, Atlanta, Georgia, 30 June–2 July 1989.

forensic sample used as evidence, rather than to identify defendants. For a further discussion of some of the statistical issues raised by the forensic use of genetic fingerprinting, see ref. 11.

It is unfortunate that the use of DNA technologies has been the subject of recent adverse publicity. In the view of Sensabaugh, the evaluation and eventual widespread acceptance of these technologies in the courtroom will depend on the

accumulation of a body of experience, and the recognition and acknowledgement of potential problems and pitfalls. An outstanding need is for agreement on when to declare a given test uninformative or sufficiently ambiguous to be unsafe as evidence. In the meantime, it is reassuring to see that agencies such as the FBI are aware of the issues and are taking steps to minimize their problems. □

Rory Howlett is an assistant editor of *Nature*.

1. Jeffreys, A.J., Wilson, V. & Thein, S.L. *Nature* **318**, 76–79 (1985).
2. Lander, E.S. *Nature* **339**, 501–505 (1989).
3. Budowle, B., Baechtel, F.S., Giusti, A.M. & Monson, K.L. *Clin. Biochem.* (in the press).
4. Budowle, B. & Baechtel, F.S. *Appl. theor. Electrophoresis* (in the press).
5. Budowle, B., Baechtel, F.S. & Adams, D.E. *Am. chem. Soc. Symp. Series* (in the press).
6. Budowle, B., Wayne, J.S., Shutler, G.G. & Baechtel, F.S. *J.*

- forensic Sci.* (in the press).
7. Saiki, R.K. *et al. Science* **230**, 1350–1354 (1985).
8. Allen, R.C., Graves, G. & Budowle, B. *Bio Techniques* (in the press).
9. Higuchi, R., von Beroldingen, C.H., Sensabaugh, G.F. & Erlich, H.A. *Nature* **332**, 543–546 (1988).
10. Budowle, B., Adams, D.E., Comey, C.C. & Merrill, C.R. in *New Directions in Forensic Science* (in the press).
11. Evett, I.W., Werrett, D.J. & Gill, P. *Nature* **340**, 435 (1989).

MATERIALS SCIENCE

Aluminium-based glassy alloys

Robert W. Cahn

A new family of glassy alloys based on aluminium, announced by three independent groups^{1–3} over the past two years, is eliciting much interest. Being both strong and ductile, the alloys may succeed in technical applications where other metallic glasses have disappointingly failed. Furthermore, as described recently by Dubois and colleagues², these glasses do not fit familiar theories and their compositions sometimes resemble those of the remarkable quasicrystals discovered five years ago, prompting important new scientific questions.

Metallic glasses (or glassy alloys) have been studied intensively since their discovery in 1960 by Duwez and his collaborators. The term 'glass' is used in preference to 'amorphous solid' to denote the fact that Duwez made his revolutionary materials by rapid cooling from the melt: the term 'glassy alloy' is preferable to 'metallic glass' because all such glasses have to be alloys as pure metals cannot be made glassy — crystallization occurs even for cooling rates as high as 10^8 °C s⁻¹. Whatever the nomenclature, the examples studied during the past three decades were almost all made of transition metals, alloyed either with other transition metals or with metalloids such as boron, phosphorus and silicon⁴. Apart from a few offbeat curiosities such as calcium-magnesium glasses and transition-metal-beryllium combinations, no other types of glass have been found — until recently.

The driving forces behind the discovery of the new alloys were first, old-fashioned scientific curiosity and second, the need to find glassy alloys whose mechanical strength can be exploited. Unlike the brittle oxide glasses widely used in glass-reinforced plastics, glassy alloys are not only very strong but also often ductile, yet

repeated attempts to use them in ribbons to reinforce composite materials have come to nothing, almost certainly because they have been too dense. The only exception is the family of Ti–Zr–Be glasses whose application foundered on the toxicity of beryllium.

Over the past 17 years, small volume fractions of glassy phase have been observed in various binary aluminium alloys on rapid solidification, notably in Al–Cu, Al–Ge and Al–Cr. Inoue *et al.*⁵ were the first to turn an aluminium alloy specimen completely to glass: some of their (Fe,Co,Ni)–Al–B glassy alloys had Al contents of over 50 per cent. A little later, Shingu and colleagues examined a series of Al–Fe–Si alloys⁶, but these were very brittle and the investigation was dropped. Only one of their alloys, Al_{69.6}Fe_{13.0}Si_{17.4} (which is close to that of the crystalline compound Al₇Fe₂Si₃), was completely turned to glass by melt-spinning (the solidification of a jet of molten alloy on contact with a spinning copper wheel). Note that the two auxiliary constituents are familiar from other glasses.

This discovery impressed the French metallurgist, Dubois, working at Nancy, who sought to predict glass-forming ranges in aluminium alloys from structural first principles, an approach he termed the 'chemical twinning model'^{9,10}. An essential part of the model is that glass formation is most likely at compositions close to those of stable crystalline compounds — a notion consistent with Gaskell's¹¹, that the disposition of nearest-neighbour atoms in glassy alloys is similar to that in the unit cell of a crystalline phase. Dubois and colleagues studied¹² the short-range order in the Al–Si–Fe glass discovered by Shingu and colleagues, finding an atomic arrangement very similar to a stable phase of

Al₇Cu. Two other groups, Masumoto's at Tohoku University and Shiflet's at the University of Virginia, independently began to investigate aluminium-rich glasses, in particular their composition ranges, mechanical properties and short-range structural order. There was especial interest in achieving the highest possible percentage of aluminium, as this would assure glasses of low density.

Behind Dubois's priority in this 'race', there lies an interesting twist. Dubois and Le Caër enclosed a paper in a *pli cacheté*, a sealed envelope, and deposited it with the French Academy of Sciences on 7 June 1982. It was opened two years later, at the authors' request, and a summary¹ was submitted to and published by the Academy in 1985. It emerged later² that the authors had joined forces with the industrial laboratory of Pechiney, the aluminium company, and had been taking out patents since 1982. The first patent discussed a wide range of glass-forming aluminium-rich compositions of up to five components, for example, Al₆₉Cu₁₇Fe₁₀Mo₃Si₃. The delay in publication achieved by the popular French device of the *pli cacheté* was probably needed because further patent applications were underway: indeed, an American patent was granted in late 1987.

Dubois's team sought to compare its first glasses, based on Al–Cu–Ni, to the recently discovered quasicrystals, most of which form in Al-rich compositions with transition metals. It turns out that those mixtures of aluminium and transition metals that are most apt to give quasicrystals are also those that require the most silicon to form glassy alloys instead (Fig. 1). The authors have, so far, pursued the matter no further, concluding in their latest paper² that "aluminium glasses inspire intellectual modesty and aesthetic contemplation in equal measure". This appealing reaction would also be apposite for recent work by Shiflet and colleagues¹³, who find that Al–Fe–Gd alloys which can form glasses, in their equilibrium state have no eutectic (a configuration in which the melting point is most strongly depressed) although this is the usual feature

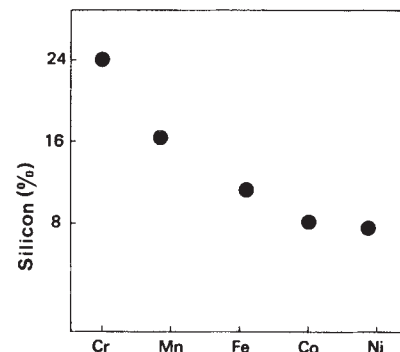


FIG. 1 Minimum concentration of silicon needed to induce glass formation in aluminium alloys. (From ref. 2.)

favouring glass formation.

Observations of rapidly quenched $\text{Al}_{86}\text{Mn}_{14}$ suggested that the product is quasi-microcrystalline, raising the problem of distinguishing such a structure from true glassiness. This distinction was achieved ingeniously by a novel calorimetric method recently designed for the purpose at Harvard¹⁴; it would be interesting to apply this technique to characterize some of the new Al-rich glasses.

The relationship between tendencies to form quasicrystals and glasses in aluminium, identified by the French team, is complemented by the Japanese efforts to interpret the role of atomic sizes and the strength of bonds between different atom pairs in influencing the ease of glass formation. (The claim² that only the French investigated the fundamentals does not really stand up to examination.) The Tohoku team, renowned for its systematic investigations of metallic glass systems, has discovered the usefulness of rare-earth metals as alloying constituents in aluminium-rich glasses; these additives are so potent that binary and ternary alloys containing up to 90 per cent aluminium form glasses. The binary glasses (Al-Y, Al-La and Al-Ce) are difficult to make and the acceptable composition ranges are narrow, but ternary compositions (Al-Y-M and Al-La-M, with M=Fe, Co, Ni or Cu) form glasses over wide compositional ranges (Fig. 2).

The tensile strength of these materials can be formidable — often exceeding 800 megapascal (or even 1,000 MPa in the case of Al-Y-Ni³ and Al-Ni-Zr¹⁵). Ternary aluminium glasses containing transition and rare-earth metals (especially Ce, Y and Gd) studied by Shiflet and colleagues, have tensile strengths up to 940 MPa. The crucial point is that these strong glasses are also very ductile, so that they are very tough (impact-resistant). Their maximum strengths are twice those available from the best age-hardenable aircraft alloys, and could be exploited in ribbons or wires for reinforcing composites, in surface

coatings or in load-bearing structures. Also, the glasses are stable against crystallization up to temperatures ranging as high as 520 °C, a final point favouring their commercial exploitation. □

Robert W. Cahn is in the Department of Materials Science and Metallurgy, University of Cambridge, Pembroke Street, Cambridge CB2 3QZ, UK.

1. Dubois, J.-M. & Le Caër, G. *Compt. Rend. Acad. Sci.* **301**, 73–78 (1985).
2. Bechet, D., Regazzoni, G. & Dubois, J.-M. *Pour la Science*, No. 139, 30–39 (May 1989).
3. Inoue, A., Ohtera, K., Tsai, A.-P. & Masumoto, T. *Jap. J. appl. Phys.* **27**, L280–L282; L479–L482; L736–L739 (1988).
4. Tsai, A.-P., Inoue, A. & Masumoto, T. *Metall. Trans.* **19A**, 1369–1371 (1988).

5. He, Y., Poon, S.J. & Shiflet, G.J. *Science* **241**, 1640–1642 (1988).
6. Cahn, R.W. in *Physical Metallurgy* (eds Cahn, R.W. & Haasen, P.) 1778–1852 (North Holland Physics, 1983).
7. Inoue, A., Kitamura, A. & Masumoto, T. *J. mater. Sci.* **16**, 1895–1908 (1981).
8. Suzuki, R.O., Komatsu, Y., Kobayashi, K.F. & Shingu, P.H. *J. mater. Sci.* **18**, 1195–1201 (1983).
9. Dubois, J.-M., Le Caër & Dehghan, K. in *Rapidly Quenched Metals*, (ed. Warlimont, H.) 197–202 (Elsevier, 1985).
10. Dubois, J.-M. *J. Less-Common Metals* **145**, 309–326 (1988).
11. Gaskell, P.H. *J. Non-Cryst. Solids* **32**, 207 (1979).
12. Dubois, J.-M., Dehghan, K., Chieux, P. & Laridjani, M. *J. Non-Cryst. Solids* **93**, 179–189 (1987).
13. He, Y., Poon, S.J. & Shiflet, G.J. *Scripta metall.* **22**, 1813–1816 (1988).
14. Chen, L.C. & Spaepen, F. *Nature* **336**, 366–368 (1988).
15. Tsai, A.-P., Inoue, A. & Masumoto, T. *J. mater. Sci. Lett.* **7**, 805–807 (1988).
16. Inoue, A. & Masumoto, T. in *Second Supplementary Volume of the Encyclopaedia of Materials Science and Engineering* (ed. Cahn, R.W.) (Pergamon, Oxford, in the press).

DNA STRUCTURE

Curves with a function

Andrew Travers

A CRUCIAL step in the enzymatic manipulation of DNA in such important biological processes as transcription, site-specific recombination and the initiation of DNA replication is the local unwinding of DNA within a short region of one to two double-helical turns. This process is frequently preceded by the assembly of a large nucleoprotein complex in which the DNA is tightly wrapped, often as a left-handed superhelix, around a protein core. The constraints on the topology of the DNA within such complexes would be expected to require that both the magnitude and direction of curvature of the path of the double helix are strictly specified. In principle, such a curved path can be imparted to a DNA molecule either by a protein bound to a bendable and flexible DNA sequence, or by the intrinsic bending conferred by particular oligonucleotide sequences — the conformationally rigid oligo (dA)-(dT) tracts^{1–3}. Yet, although many examples of protein-induced and intrinsic DNA bending have been reported, it is only now that clear evidence of the functional significance of the curvature as such is emerging. Three new papers^{4–6} emphasize the importance of this aspect of the biological function of DNA.

One large condensed nucleoprotein structure that lends itself to experiments designed to address this question is the intasome⁷. This complex mediates the integration of viral (phage λ) DNA into the bacterial chromosome and contains several molecules of two DNA-binding proteins, the phage-encoded integrase (Int) and the *Escherichia coli* integration host factor (IHF). When bound separately to their respective binding sites, IHF, but not Int, induces a substantial DNA bend^{8–10}, estimated¹⁰ to be at least 140°. One possible function of IHF would be to

bend the DNA and thus define the three-dimensional path of the double helix in the intasome; in other words, IHF would act as an architectural element.

This concept has been tested by Landy and his colleagues, who have recently shown¹¹ that the bivalent Int protein can simultaneously bind to two different segments of *attP*, the genetically defined attachment site on the phage DNA. These segments are separated by a DNA loop whose trajectory is defined by bound IHF. For this trajectory to be strictly defined in the intasome, the bends introduced by the three component IHF molecules must be correctly phased relative to each other: a one-base-pair change in the separation between two binding sites would alter the relative direction of the bends at the two sites by 34° and substantially alter the three-dimensional path of the double helix. On page 255 of this issue⁴, Snyder *et al.* show, by varying the spacing between pairs of IHF-binding sites in *attP* that, even in the absence of Int, optimal IHF binding induces bending that would direct *attP* DNA to follow a left-handed superhelical path. This result is consistent with the earlier observation that intasome formation is facilitated by negative supercoiling^{12,13} and confirms the importance of

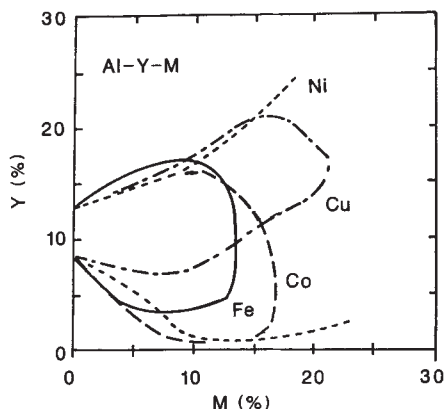


FIG. 2 Binary and ternary aluminium alloys whose composition fall inside the loops are able to form glasses if rapidly quenched. (From ref. 16.)

100 years ago

From the British Association

As General Strachey, late President of the Royal Geographical Society, remarks: "The reflection can hardly be avoided that, great as has been the advance of exploration in Africa during the last twenty or thirty years, the interest of geographers will be more and more centred in that continent. Excluding the polar regions, there is no considerable portion of the earth's surface, unless it is in Africa, the essential outlines of which have not been delineated." From *Nature* **40**, 494; 19 September 1889.

IHF as an architectural element.

To test directly whether the DNA bends themselves are crucial for intasome function, Goodman and Nash adopted the strategy of 'bend swapping', as also reported in this issue⁵. When they replaced one IHF-binding site in *attP* by a site with high affinity for CRP (or CAP) — a DNA-binding protein that is unrelated to IHF and which functions normally to mediate cAMP-induced transcription — they found that CRP bends this site to an extent comparable to that of IHF. In such constructs, significant recombination was observed only in the presence of CRP, suggesting that only the bending of DNA and not additional heterologous protein-protein contacts are necessary for the assembly of a functional intasome. This conclusion is confirmed by the finding that an intrinsically bent DNA sequence, with a bend of approximately 120°, can also substitute for the IHF-binding site provided that the bend is appropriately phased. Together these results establish that the principal — and possibly the sole — *in vivo* role of IHF is to bend DNA at specific sites. Such a role would be fully consistent with its multifarious effects on transcription, recombination and DNA replication.

The 'bend swap' experiments establish that, in the intasome, the determinant of biological function of intrinsically bent DNA is its overall curvature, rather than the associated sequence-dependent variations in local DNA conformation. The efficacy of curved DNA in substituting for functions normally mediated by sequence-specific DNA-binding proteins extends to the activation of transcription initiation by *E. coli* RNA polymerase. In the complex formed between the polymerase and the promoter, the DNA is inferred to be wrapped around the protein for up to 60 base pairs upstream of the transcription start point. In many promoters the DNA upstream of a highly conserved hexamer, located 35 base pairs before the start point, is often intrinsically bent. In a recent study of the functional significance of this bending Achtberger and McAllister⁶ have shown that promoter activity is exquisitely sensitive to the direction of curvature in this region. Unexpectedly, when correctly phased bent DNA is placed 60–90 base pairs before the start point, beyond the previously observed limit of binding of a single polymerase molecule, transcriptional activity is conserved. This observation suggests either that the curved DNA serves simply to direct the polymerase to the promoter region, or that the curve allows the DNA to bend back to make additional contacts with the enzyme.

In a related set of experiments, Bracco *et al.*¹⁴ demonstrate that the requirement for CRP for the *in vivo* activation of the *E. coli* *gal* promoter can be overcome by

replacing the CRP-binding site by curved DNA. Again, the extent of transcriptional activation is strongly dependent on the orientation of the inserted curve, to the extent that a change of approximately 180° in the direction of bending moves the transcription start point half a double-helical turn upstream — from the *gal* P1 start to the P2 start. This result is fully consistent with the notion that the bending of DNA by CRP is a necessary component of the activation process¹⁵.

The principal conclusion from the studies on both the intasome and RNA polymerase is that the bending of DNA is an important determinant of both the function and the architecture of large nucleoprotein complexes. Interestingly, although it is crucial in both complexes to conserve both the direction and magnitude of bending, the experimental results indicate that the precise length of DNA accommodated by the complexes can vary, albeit in a measured manner. This suggests that the protein components of the complexes are themselves sufficiently flexible to accommodate such variations. A further consequence of this flexibility would be that the topology of the DNA within such complexes would be largely

conserved. Thus, each complex would constitute a topological micro-domain delimited by protein contacts towards the flanks of the binding site. An important question remaining is whether the left-handed writhing created by the bending is converted into local limited untwisting and, if so, how. □

Andrew Travers is in the Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK.

1. Wu, H.-K. & Crothers, D.M. *Nature* **308**, 509–513 (1984).
2. Travers, A. & Klug, A. *Nature* **327**, 280 (1987).
3. Calladine, C.R. & Drew, H.R. *J. molec. Biol.* **192**, 907–918 (1986).
4. Snyder U.K., Thompson, J.F. & Landy, A. *Nature* **341**, 255–257 (1989).
5. Goodman, S.D. & Nash, H.A. *Nature* **341**, 251–254 (1989).
6. Achtberger, C.F. & McAllister, E.C. *J. biol. Chem.* **264**, 10451–10456 (1989).
7. Echols, H. *Science* **233**, 1050–1056 (1986).
8. Prentki, P., Chandler, M. & Galas, D.J. *EMBO J.* **6**, 2479–2487 (1987).
9. Robertson, C.A. & Nash, H.A. *J. biol. Chem.* **263**, 3554–3557 (1988).
10. Thompson, J.F. & Landy, A. *Nucleic Acids Res.* **16**, 9687–9705 (1988).
11. de Vargas, L.M., Kim, S. & Landy, A. *Science* **244**, 1457–1461 (1989).
12. Spengler, S.J., Stasiak, A. & Cozzarelli, N.R. *Cell* **42**, 325–334 (1985).
13. Griffith, J.D. & Nash, H.A. *Proc. natn. Acad. Sci. U.S.A.* **82**, 3124–3128 (1985).
14. Bracco, L. *et al.* *EMBO J.* (in the press).
15. Buc, H. *Biochem. Soc. Trans.* **14**, 196–199 (1986).

A chip off the new technology block

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

A pseudo-colour scanning electron micrograph of a 64-K bit memory chip. This voltage-contrast image (55 × 40 μm) shows the paths of applied voltage in yellow. This year marks the 150th anniversary of the founding of the Royal Microscopical Society. To celebrate 'the year of the microscope', the Science Museum, Exhibition Road, London, is mounting the first major interactive display of light and electron microscopy ever staged. Visitors

will be able to observe their own specimens and operate a wide variety of microscopical equipment — including scanning (SEM) and transmission (TEM) electron microscopes. Displayed alongside the most advanced equipment available to modern-day microscopists, will be a sequence of instruments and images illustrating the history of microscopy since the Society was formed. The exhibition runs until January 1990.

Claire Hooper

Trying to count baryons

Craig J. Hogan

ACCORDING to Eddington, shortly before he died, the number of protons and electrons in the entire Universe is $\frac{3}{2} \times 136 \times 2^{256}$. Although many now would doubt that he was exactly right, the quantity of matter is still a question that provokes strong opinions and prejudices because of its prime significance in models of the Big Bang. How much cosmic matter there is and what forms it takes were the subject of a recent meeting* on *Baryonic Dark Matter*.

Why "*Baryonic*"? This term refers to the baryons — neutrons and protons — that contribute most of the mass of all ordinary matter. The issue is how much of the mass of the Universe is made of such familiar stuff, perhaps in an unfamiliar form, and how much is made of something completely different, for example, an unknown exotic particle left over from the early Universe. The study of baryonic dark matter brings together diverse areas of astronomical research, from comets and asteroids and interstellar matter to intergalactic gas and the physics of the Big Bang. I will focus here only on the narrower question of how much dark baryons can contribute to the mean density of the Universe.

Why "*Dark Matter*"? We now know empirically from the dynamics of galaxies, galaxy clusters and superclusters (from the requirement that matter with known velocities be gravitationally bound to systems of known size) that most of the matter in the Universe is dark — it emits light too weakly to be detected. Nevertheless, fairly firm lower limits on the mean baryon density are available from several different points of view. For example, cold diffuse gas can be seen because it *absorbs* light (W. Sargent, California Inst. Technology); indeed observations of absorption lines in the spectra of quasi-stellar objects (QSOs) give one of the firmest lower limits on the density of dark baryons (although because these are seen at substantial redshifts, the same gas might not be dark today).

Neutral hydrogen

Dozens of QSOs are known at high redshift, and their sightlines traverse a known pathlength through the Universe. About one in five passes through a cloud of matter of very high neutral-hydrogen column density — high enough for the amount of hydrogen to be accurately measured. The physical state of the clouds is somewhat uncertain — they may be gaseous dwarf galaxies or giant gas-rich disks — but their contribution to the mean

density is estimated directly from the observations, by dividing the total column density by the total line-of-sight.

Cosmic density is best expressed in special units, using the cosmic density parameter Ω . $\Omega = 1$ corresponds to the density needed to balance exactly the kinetic energy of expansion and gravitational potential energy, giving the Universe zero total energy. (Relating this to a physical density involves knowing the expansion rate in terms of Hubble's constant $H = 50h \text{ km s}^{-1} \text{ Mpc}^{-1}$; h lies between 1 and 2.) A density very close to this critical value is not only predicted by inflation models of the Big Bang but is always expected unless the present epoch is somehow imprinted at the moment of the Big Bang. Yet direct measures of baryonic density, Ω_b , always give very small values; the absorption clouds at a redshift of 2 and present-day luminous stellar populations in galaxies (which may be the same material) each contribute only about $5 \times 10^{-3} h^{-1}$.

A less direct but precise fix on the total baryon density can be obtained by matching the predicted abundances of various elements, which depend on Ω_b , with observations (B. Pagel, Royal Greenwich Obs.). The simplest homogeneous Big Bang model can simultaneously fit the best current estimate of the primordial abundances of several light elements only for a single density, $\Omega_b h^2 = 0.04$, and 95 per cent confidence limits on these abundances still permit only a narrow range of density: $0.04 \leq \Omega_b h^2 \leq 0.06$. This range has steadily narrowed as observations have improved: the strictest upper limits come from very accurate determinations of helium in gas-rich dwarf galaxies, the lower limits from the lithium in atmospheres of old stars and interstellar measurements of deuterium and helium-3.

If we take this model literally (and its success in fitting diverse abundances suggests that we should) then we can draw several interesting conclusions. First, about 90 per cent of the baryons that exist have not been detected either by absorption or emission and must exist in some currently dark inert form, the most conservative candidate being some form of burned-out stellar remnants (degenerate dwarfs, neutron stars or black holes). This idea can be squared with models of chemical enrichment and evolution of galaxies (a popular theme at the meeting) and models for forming the submillimetre radiation background (S. Hayakawa, Nagoya Univ.). Alternatively, it may be in the form of 'jupiters' or 'brown dwarfs' — bodies too small to burn brightly as main-sequence stars (B. Carr, Queen Mary

College; L. Nelson, Canadian Inst. Theoretical Astrophysics). Such bodies would have no appreciable effect on chemical enrichment. Another interesting possibility (D. Lynden-Bell, Inst. Astronomy, Cambridge) uses even smaller degenerate bodies, hydrogen icebergs, but it is harder to see how to form these.

Second, there must also be *some* non-baryonic dark matter, because the mean density of all dark matter is known to contribute at least $\Omega \approx 0.1$, as measured from the dynamics of various galactic cluster and supercluster systems (this conclusion could not be drawn before these new abundance data were available). Finally, if Ω really is very close to 1 as we would like to think, cosmic matter is almost all (≥ 94 per cent) nonbaryonic. (Certain theories of galaxy formation also lend support to the idea of a critical density of exotic matter, because the gravitational instabilities needed to break up the primordial Universe can occur in inert dark matter without creating excessive anisotropy in the cosmic background radiation; J. Beckman, Inst. Astrophysics, Tenerife; J. R. Bond, Canadian Inst. Theoretical Astrophysics.) These three conclusions form the orthodox view on the subject.

Modifications

But in spite of its broad success in explaining the pattern of primordial abundances, perhaps one should not believe the standard homogeneous Big Bang model in literal detail. Large-scale lumps of matter, for example, could have collapsed at the period of recombination, when nuclei and electrons first formed atoms, locking up all the 'wrong' abundances in remnants that never mix with observed material. More plausible small-scale departures from homogeneity, such as might be expected from a cosmic phase transition from quark to hadronic matter, occurring at a temperature several thousand times that for nucleosynthesis, would create neutron-rich pockets of matter at nucleosynthesis. The effect leads to a modification of the abundance predictions that reproduces the observed abundance pattern over a wider range of baryon density than in the standard model — perhaps even up to the critical density. The attraction of this idea is that no exotic matter would be required.

When strong-interaction physics is better understood, such modifications to the simplest Big Bang might actually become mandatory; present theories are mixtures of calculation and conjecture. Modelling of these universes is, however, gradually moving closer to realism (W. Fowler, California Inst. Technology; K. Sato, Tokyo Univ.). Supercomputers are being used to compute particle diffusion and nuclear reactions both realistically and simultaneously; new laboratory experiments must be performed to measure

* NATO Advanced Study Institute *Baryonic Dark Matter*, Cambridge, UK, 17–28 July 1989.

ordinarily unimportant reaction rates that dominate in a neutron-rich environment. New species are emerging that may be cosmologically prominent: primordial beryllium and even heavier elements are now being predicted to occur in observably copious quantities. Current indications are that such models may achieve $\Omega = 1$ only over very narrow ranges of parameters, but much theoretical refinement remains to be done.

The excitement now is that nuclear abundances might eventually be used to probe not just the mean density of baryons, but also events at a much earlier time — not just the first three minutes, but even the first three microseconds, when the Universe turned from quark soup into ordinary hadronic plasma. □

Craig J. Hogan is at the Steward Observatory, University of Arizona, Tucson, Arizona 85721, USA.

ARCHAEOLOGY

New Bronze Age civilization

Paul Bahn

A book published privately by the Ligabue Study and Research Centre¹, has brought to light the recent discovery of a distinctive civilization of the Bronze Age (late 3rd to early 2nd millennium BC), contemporary with those of Mesopotamia, Egypt and the Indus. Until the discovery, in the semi-desert steppe region of northern Afghanistan known as Bactria, it had been thought that the eastern area of Central Asia was uninhabited through the Bronze Age, entering the historical record only as a part of the Persian Empire in the 5th century BC.

In the mid-1970s, however, a great abundance of objects appeared on the antiques market in the Middle East and Europe that came from clandestine excavations in northern Afghanistan. These represented the first definite evidence of a civilization which, at that stage, could not be pinpointed in space or time. All that was known was that the items — bronze, gold and silver, and stones such as calcite and chlorite, and semi-precious stones like lapis-lazuli, turquoise and carnelian — had been plundered by villagers from burial sites in southern Bactria; and that

many were comparable to, albeit very distinctive from, objects of the great civilizations of the 3rd and 2nd millennia BC in Egypt, Mesopotamia and the Indus. Certain items were acquired by important museums such as the Louvre² and New York's Metropolitan Museum of Art³, but many went to private collections.

This richness in metals and fine statuary revealed that Bronze Age Bactria had hitherto unsuspected wealth, as well as technological and artistic achievements. It aroused the interest of Soviet scholars, and led to a Soviet-Afghan expedition that explored the southern Bactrian Plain from 1969 to 1979, under the leadership of Viktor Sarianidi, director of the Moscow Institute of Archaeology. Their surveys and excavations led to the discovery of numerous important sites^{4,5}.

Sarianidi saw many tombs from which the rich grave goods had already been plundered; they appeared to be catacomb pits with niches at their base that originally contained the objects. The placing of portable wealth in tombs is characteristic of the Central Asian Bronze Age, whereas in Mesopotamia and other contemporary

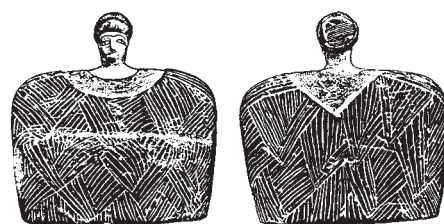


FIG. 2 Composite female statuette in chlorite and white limestone, about 10 cm high.

civilizations, tombs that are not royal contain few such items.

Perhaps the most important discovery to have been made so far is the architectural complex of the Dashly Oasis in northern Afghanistan. Two major structures, dubbed the 'palace' and the 'temple', have been uncovered at the site of Dashly 3. The former comprises a large rectangular walled area, 84 × 88 m, which encloses a central court measuring 38 × 40 m. It was clearly built as a planned complex, using bricks of standardized size, and its geometric layout, so different to the architectural use of space in the Indus, Iran and Mesopotamia, is echoed in the designs of the metal compartmented seals that seem to characterize the Bactrian Bronze Age. The 'temple', on the other hand, is a circular structure some 40 metres in diameter with nine towers around its perimeter (Fig. 1), and may have been defensive: certainly the Bactrian Bronze Age is distinguished by the large number of fortress sites located within towns containing elaborate structures.

An alternative interpretation is that Dashly's 'palace' is in fact a market place or bazaar, and its 'temple' a caravanserai⁶. That would certainly fit with the likelihood that the Bactrians were first and foremost nomads; their seals bear depictions of tethered and harnessed camels, as well as cattle, sheep and goats. Their region was a kind of emporium for international trade, serving as a vector and link between different cultures and laying the bases for the great caravan routes that would join Asia and Europe for thousands of years. It held the monopoly on commercial traffic along these routes, and those controlling the flow were able to amass concentrations of the precious and prestige goods that were to lead to their civilization being discovered by archaeologists.

As not much work has yet been done, we still know very little of the social organization in Bronze Age Bactria, or of its political or economic system. This was not, however, Mesopotamia in Central Asia; Bactria did not develop into an integrated state, and it never produced writing. It remained a cross-roads of trade routes, not a stable form of civilization.

The new book¹ has brought together a series of essays by leading specialists as well as a superb photographic archive of Bronze Age Bactrian objects, many of

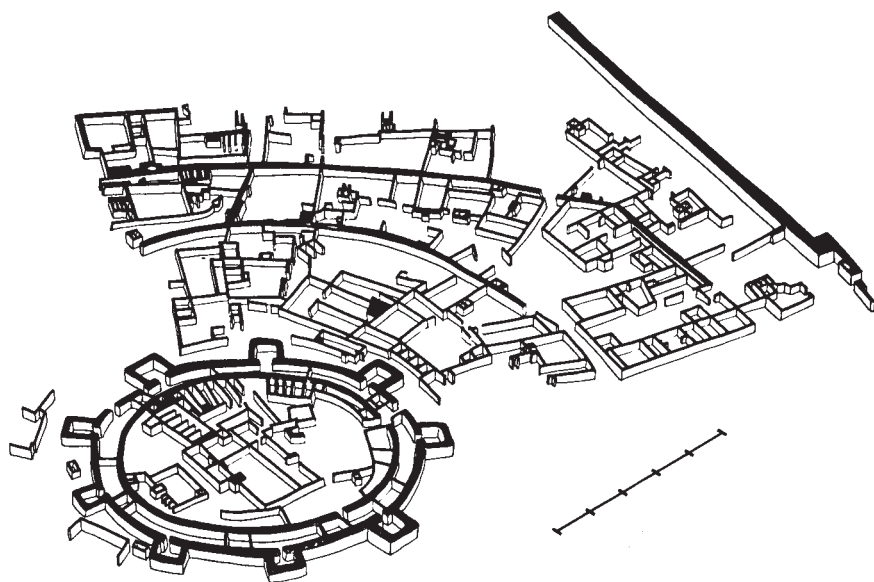


FIG. 1 Axonometric view of the 'Temple' at Dashly 3 (after Sarianidi). (Scale bar 25 m.)

them in private collections and derived from the chaotic and uncontrolled period of clandestine excavation. They include seals, vessels of stone and metal, pottery, and an astonishing variety of metalwork, most of it apparently non-utilitarian, including animal figurines, spectacular axes decorated with animals, and large pins with animal or geometric heads. Perhaps the most striking objects are the composite statuettes in chlorite and white limestone (Fig. 2).

Distinctive as it is, this material nevertheless shows plainly the influence of surrounding cultures, underlining the interrelated system that covered the whole of the Middle East at the time, linking the world of the Indus with the Iranian

Plateau and the Sumerian city states. It is to be hoped that archaeological activity will soon be able to recommence in Afghanistan, so that Soviet and other scholars can learn more about the Bactrian civilization. □

Paul Bahn is a freelance writer on Archaeology at 428 Anlaby Road, Hull HU3 6QP, UK.

1. Ligabue, G. & Salvatori, S. (eds) *Bactria: an Ancient Oasis Civilization from the Sands of Afghanistan* (Erizzo, Venice, 1988).
2. Amiet, P. *L'Age des Echanges Inter-Iraniens, 3500-1700 avant J-C* (Editions de la Réunion des Musées Nationaux, Paris, 1985).
3. Pittman, H. *Art of the Bronze Age — SE Iran, W. Central Asia, and the Indus Valley* (Metropolitan Museum of Art, New York, 1984).
4. Sarianidi, V. *Die Kunst des alten Afghanistan* (Leipzig, 1986).
5. Sarianidi, V. in ref. 1, p. 107.
6. Lamberg-Karlovsky, C.C., in ref. 1, p. 13.

ECOLOGY

Upwardly mobile roots

Peter D. Moore

STRANGLER figs of tropical forests have a certain macabre fascination, perhaps because their lifestyle is more overtly aggressive than that of most plants. They begin life in the canopies of host trees, germinating in pockets of humus formed by epiphytes and litter on branches, in leaf bases and in junctions with the trunk. Unlike other epiphytes, the maturing figs establish contact with the ground by the development of long twining roots; their subsequent growth may totally eclipse that of the host and result in its death. Starting life as an epiphyte has the advantage that light levels are higher than at the forest floor, and the depredations of animals and fungi may also be avoided. They also benefit from the higher nutrient levels found in the canopy humus pockets, according to recent work on the strangler figs *Ficus pertusa* and *F. trigonata* in Venezuela by Putz and Holbrook¹. The figs may even produce upwardly growing roots from the ground so that they can continue to tap this elevated nutrient resource after contact with the soil is established.

One might suppose that nutrients such as nitrogen, phosphorus, potassium and magnesium would be in short supply for an epiphytic plant. Suspended above the influence of the soil supply, such a plant is essentially dependent on nutrient input from precipitation, together with some recycling from the decomposition of trapped leaf litter and perhaps some fixation of nitrogen by cyanobacteria, either free-living or as lichen symbionts. But there is also a nutrient supply from the leaching of the canopy leaves. In the case of temperate woodlands, it has long been recognized that a very significant proportion of the canopy to soil nutrient movement takes place via such leaching processes², and studies of the Spanish

moss (*Tillandsia usneoides* — a bromeliad epiphyte of the south-eastern United States) have demonstrated³ a close dependence of the epiphyte on this supply of nutrients. Trees with easily leached

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Deadly embrace of the Florida strangler fig, *Ficus aurea*.

canopies (such as pondcypress, *Taxodium distichum*) are richer in Spanish moss than those with low canopy leaching (such as slash pine, *Pinus elliotii*). So nutrient supply of this type may limit the establishment and growth of epiphytes.

The nutrient resource that lies in the epiphytic ecosystems of the canopy may be tapped by the very trees that support them. Nadkarni reported⁴ that many host trees in both temperate and tropical rain forests exploit the organic debris of their epiphyte communities by producing

adventitious roots from their branches below the epiphyte mat. This may be a particularly important source of nutrients if the forest soil is poor, as in the case of the temperate rain forests of Washington State on the oceanic western coast of North America, where vine maple (*Acer circinatum*) and red alder (*Alnus rubra*) have particularly heavy epiphyte loads and well-developed canopy roots.

But tapping the canopy nutrient reservoir may go one step further. Sanford has described⁵ tropical forest trees in the Amazon basin with apogeotropic roots. These emerge from the soil, grow up the trunks of neighbouring trees and tap into the nutrients leaching from their canopies. To test the role of nutrients in the development of these roots, Sanford built artificial columns topped with 'baits' consisting of forest litter or manure, and some empty controls. The most extensive development of apogeotropic roots was found on the manure-topped structures and the least on the controls, showing that the roots do indeed grow upwards in response to nutrient supply.

The work of Putz and Holbrook¹ on Venezuelan strangler figs growing on a host palm, *Copernicia tectorum*, shows that they too produce apogeotropic roots, so ensuring that they remain in contact with the organic pockets in the canopy even when they have an independent, ground-based root system. The suspended 'soil' in these pockets was found to be richer in nitrogen ($\times 3$), potassium ($\times 6$) and magnesium ($\times 1.5$) than the soil beneath the trees, so the rewards of climbing back up the tree are apparent. A further interesting observation is the absence of vesicular arbuscular mycorrhizal infection in the epiphytic roots. It is unknown whether this is because such infection would be of little benefit to the plant or because physical difficulties prevent fungal infection.

The strategy of beginning life as an epiphyte therefore seems to have clear nutritional advantages which may persist in later life by the development of upwardly mobile roots. But this will prove of value only if the host tree survives beneath its fig load. The quality of mercy, we are told, droppeth as the gentle rain from heaven, in which case the strangler fig might well benefit from exercising a little of the same and thus be assured of a continued supply of nutrients from the canopy of its reprieved host. □

Peter D. Moore is in the Division of Biosphere Sciences, King's College, London W8 7AH, UK.

1. Putz, F.E. & Holbrook, N.M. *Am. J. Bot.* **76**, 781-788 (1989).
2. Carlisle, A., Brown, A.H.F. & White, E.J. *J. Ecol.* **54**, 87-98 (1966).
3. Schlesinger, W.H. & Marks, P.L. *Am. J. Bot.* **64**, 1254-1262 (1977).
4. Nadkarni, N.M. *Science* **214**, 1023-1024 (1981).
5. Sanford, R.L. *Science* **231**, 1062-1064 (1987).

Spreading the word

David Baulcombe and Roger Hull

A SUBJECT that is attracting increasing attention among both plant virologists and cell biologists is the movement of viruses through plants. Progress in that area was evident at several recent meetings*, which have covered various aspects of the molecular biology of plant viruses. But the same meetings have demonstrated that molecular biology has confused further some of the thornier problems of plant virus taxonomy.

Viruses move through plants either by short-distance cell-to-cell spread through plasmodesmata or by long-distance spread through the vascular system. There is increasing evidence that cell-to-cell spread is facilitated by one or more virus gene products, the most studied of which is the P30 protein of tobacco mosaic virus. Using various manipulations of an infectious cDNA clone of the virus, W.O. Dawson and K. Lehto (University of California, Riverside) showed that the nearer a gene was to the 3'-end of the genome the more strongly it was expressed but that, within the limits examined, it was not the strength but the time of expression of P30 that was important for efficient virus spread; early expression was crucial for proper function. How P30 functions is still unknown but there are various pointers. The *in vivo* expressed product is phosphorylated (D. Zimmermann and A. Haley, MRC Laboratory of Molecular Biology, Cambridge). Nicotinic acid blocks the transport of tobacco mosaic virus in leaf disks and this inhibition is overcome by cyclic AMP or by papaverin or calyculin, both of which increase cyclic AMP levels in leaves (J.G. Atabekov, Moscow State University).

Movement models

Broadly speaking, there are two models to explain how viruses move from cell to cell. In one, there is a specific interaction between the virus and the movement apparatus that allows the virus to translocate. In the alternative model, the intercellular connection is 'gated' so that abnormally large molecules, including virions or viral nucleoproteins, can pass through. So far, several lines of evidence point towards the second model, although there will be some complications. The absence of specificity in the process is suggested by the observations that some viruses assist movement of other viruses in certain hosts (Atabekov). In one example, this interaction is manifested as complementation of a defective homologue of P30 in tobacco rattle virus by intact

tobacco mosaic virus (V. Ziegler-Graff, Sainsbury Laboratory, Norwich).

Other experimental evidence for the gating model is provided by transgenic tobacco plants that express the P30 gene. In these plants the P30 protein is associated with plasmodesmata, especially in older leaves (D. Atkins, John Innes Institute, Norwich). The gating capacity of plasmodesmata in the plants was measured by video-intensified microscopy of fluorescent dyes of different sizes that had been encapsulated in liposomes and injected into the vacuole; the liposomes fused with the tonoplast membrane and released the dyes into the cytoplasm (W. Lucas, University of California, Davis). Control (non-transformed) plants did not allow cell-to-cell movement of dyes of relative molecular mass (M_r) greater than 800, whereas in the transgenic plants the gating of 11,000–17,000 M_r dyes was observed. Of course, viruses are much larger, so simple gating cannot be the whole story.

Although there is much less information about long-distance transport of viruses there is one important experiment that throws light on the involvement of the viral coat protein. T. Meshi and Y. Okada (University of Tokyo) mutated a cDNA clone of tobacco mosaic virus so as to disrupt the origin of virus assembly but to retain the coat protein sequence. This mutant, which expressed coat protein but was defective in particle assembly, spread systemically through the plant almost as rapidly as the wild-type virus. Thus, efficient assembly is not necessary for long-distance movement, though other experiments show that expression of coat protein is required.

A role of coat protein in transport of virus within the plant is also suggested from data obtained with plants transformed to express the viral coat protein. These plants are resistant to viral infection due to an action of the coat protein at several points in the infection cycle. An effect on transport is inferred from experiments in which efficient movement of virus through the stem is prevented by grafting a section of a plant expressing coat protein into the movement path of the virus (R. Beachy, Washington University, St Louis). Curiously, it was necessary to include a leaf on the grafted segment, otherwise the virus moved normally.

There is also an effect of coat-protein expression on virus disassembly, demonstrated in protoplasts of coat-protein-expressing tobacco transfected with pseudovirions — non-viral RNA, in this case glucuronidase mRNA, coupled to viral origin-of-assembly and packaged *in vitro* (T.M.V. Wilson, John Innes Institute,

Norwich). Expression of pseudovirions was inhibited in mesophyll protoplasts but not in epidermal cells. As the epidermis is where infection is established, at least for mechanically-inoculated tobacco mosaic virus, it may be necessary to invoke another level of coat-protein action in order to reconcile the mesophyll specificity with the observation that coat-protein expression on a local lesion reduces the efficiency with which viral infection is established (Beachy). Thus, the analysis of coat-protein-mediated resistance is leading to discovery of new processes in the viral infection cycle. In addition, the complex mechanism underlying the resistance suggests that this will be an effective and durable trait for use in agriculture. The acid test will be in the field under a variety of conditions, and subject to environmental acceptability.

Taxonomy

Viral taxonomy has always been a difficult area and is likely to remain so following new discoveries about the organization of RNA viral genomes. Many of the previous groupings have now been blurred by the discovery that relationships between viruses vary in different parts of the genome. For example, the three different luteoviruses sequenced are all inter-related at their 3'-ends but at their 5'-ends the relationships differ. As summarized by R. Goldbach (Agricultural University, Wageningen), the 5'-end of beet western yellows luteovirus is similar to southern bean sobemovirus; barley yellow dwarf luteovirus is similar to tobacco necrosis virus and carnation mottle virus; potato leafroll luteovirus has no obvious similarity to any of these viruses.

Another example of RNA recombination that may throw light on the mechanism of hybrid genome formation is the template switching observed in satellite RNAs of turnip crinkle tobravirus. These RNA satellite molecules comprise, in part, a true satellite RNA that is not obviously similar to the helper virus and, in other parts, remnants of the helper virus genome. It appears that the replicase, having completed synthesis on one strand, or dropped off that strand, has a propensity to re-initiate synthesis at internal sites with similarity to the terminal promoters (A. Simon, University of Massachusetts). Transcription continues contiguously with the pre-existing RNA strand, producing a mosaic of genomic RNA sequences.

Clearly, good progress has been made with the analysis of the viral side of the pathogenic interaction; one looks forward to equally good progress with the analysis of the diseased and resistant plant. □

David Baulcombe is in the Sainsbury Laboratory, Norwich NR4 7UH, and Roger Hull in the Department of Virus Research, John Innes Institute, Norwich NR4 7UH, UK.

* 5th International Congress of Virology, Kyoto, Japan, 20–27 August 1988; NATO Advanced Research Workshops, Chichester, UK, 12–16 April 1989 and York, UK, 2–7 July 1989; EMBO Workshop, Wye, UK, 16–19 July 1989.

William Shockley (1910–1989)

WILLIAM SHOCKLEY, who died on 11 August in California, must have been one of the most controversial scientists of this century. He collaborated with John Bardeen and Walter Brattain in the invention of the transistor, which has revolutionized modern life. As a driving force behind the exploitation of miniaturized electronics, he was sometimes called the father of silicon valley. But in the last decades of his life, these achievements became overshadowed by the notoriety of his views on racial differences.

It seems that, having gained the Nobel Prize for physics for his work on transistors, Shockley developed an affliction, shared by a few other laureates, that is rooted in public reverence for the prize. The victims (so far, always male) note that their opinions on almost any subject are treated with respect, even in fields in which they have no expertise; and deciding that one of these fields is dominated by stick-in-the-muds, they propose to bring them a breath of fresh air.

Shockley selected social anthropology and genetics. Human intelligence, he proposed, is inherited, and blacks are genetically inferior in intellect to whites: "genetics plays a role in the disadvantages of our nation's black minority. My research on IQ and race gives the estimate that, in negro populations with average IQs in the 70 to 90 range, each additional 1 per cent of caucasian ancestry raises average IQ by 1 per cent"¹. The latter point he made in spite of the wide margin of error in IQ measurements. Shockley proposed a "voluntary sterilization bonus plan . . . based on payments of a thousand dollars for each point below 100 IQ".

McClelland's view², that IQ measurements are "part of an elitist mechanism to discriminate against the disadvantaged" typifies the low value specialists place on IQ measurements. The allegation that any ethnic group has an hereditary lack of intelligence is contrary to measurements of human genetic variation. For example, the estimated number of gene differences between individuals from different populations (caucasian, negro, Japanese) is only slightly greater than the number between individuals from the same population³. Furthermore, the allegation goes against people's aspirations towards unity. As a result, Shockley's lectures caused near-riots at campuses.

Shockley admitted in 1965 "several intellectuals have discouraged me from publicly expressing" the ideas, but added "I have faith in the long-term values of open discussion"⁴. Although his ideas, which were not novel, were examined and rejected, he continued to advance them.

More palatable than his ethnic proposals were Shockley's views on the inheritance

of genius. The emergence of genius that transcends circumstances is well known. Examples include the mathematician Ramanujan and, in music, Mozart.

Shockley evidently favoured the idea that the genes that produce geniuses

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

William Shockley — notorious views.

should be passed on to offspring through insemination, making, at the age of 68, a personal contribution to this effort via a sperm bank in Southern California. This donation he discussed at length in an interview with *Playboy* magazine⁵. Younger donors are usually preferred because of the lower count of viable spermatozoa in older men, not to mention unsubstantiated speculation that deleterious mutations may accumulate in the germ line. (There is also the problem of genetic recombination, famously noted once by George Bernard Shaw. It is claimed that the beautiful actress Ellen Terry expressed a wish to mother a child by him that would have her looks and his brains. Shaw's diffident reply was that he feared the child might have his looks and her brains.)

Shockley argued that "the breeding of good genetic material, whether the people are rich or poor, is desirable. We want more Lincolns, not fewer", and bolstered this simplistic approach by quoting Herman Muller whose advocacy of eugenics is generally rejected, in spite of his eminence in genetics.

It is regrettable that Shockley's great contribution to the 'transistor age' is marred by his ideas on genetics. Astonishingly, he viewed⁵ his theories of 'dysgenics' as more important than his work on the transistor, and continued to publicize these ideas at the same time as another Nobel laureate, Martin Luther King, led us in the opposite direction. H. Kallikak

1. Shockley, W. *The Humanist* **32**, 16–17 (1972).

2. McClelland, D. C. *The Humanist* **32**, 9–10 (1972).

3. Nei, M. & Roychoudhury, A. R. *Science* **177**, 434–436 (1972).

4. *US News & World Report* **59**, 68 (22 November 1965).

5. *Playboy* **27**, 69–87 (August 1980).

Dental telepathy

OUR sense of touch is almost totally ignored by modern information technology, except in the special case of Braille for fingertip reading. Daedalus sees this as a lost opportunity. He points out that touch is most discriminating, not at the fingertips at all, but at the tongue. We all know how instantly the tongue detects and explores the smallest dental changes: persistently reminding us, for example, of a tiny chip lost from a tooth. Dental patients often complain of irregularities in a new filling that the dentist can hardly even see.

So Daedalus is devising a new communication device, the DREADCO Datadenture. His prototype has a datahead, resembling the printing head of a dot-matrix printer, mounted in the centre of its dental plate. A matrix of tiny pins, each capable of independent vertical travel, can present the tongue with a wide range of possible conformations. Toothless volunteers and DREADCO dentists are cooperating to discover how many configurations of the active surface can be distinguished and how rapidly, what codings are most easily learnt, and so on.

The prototype gets its power and information from a rather clumsy cable entering the mouth. The final design, more elegantly, will use magnetic induction. The primary of the drive-transformer will be mounted on the wearer's collar, and will energize the pin-actuating coils in the wearer's mouth as secondaries. Each drive-coil will be given a different resonant frequency, so that the induction link can address them all individually by simple frequency modulation. All the coding circuits and power-supply units would be safely outside the body, so that the mouth-borne data head may even be miniaturized to the size of a single tooth filling. In exchange for this much more modest dental sacrifice, the wearer would gain a wonderful private data-link, fed from information relayed to the collar induction unit by a nearby transmitter.

At first Daedalus saw his dental data-link as an exotic private paging device, perhaps calling the ambassador unobtrusively from the embassy party to cope with sudden international crises. Less romantically, he now sees it as a source of steadily updated 'background information' for working professionals: giving the time or the stock-market prices, the changing output of a plant or the speed and heading of an aircraft. The user would soon learn to 'read' it quite automatically, while coping with foreground tasks, scanning instruments or talking to colleagues. This parallel data-path would be wonderfully clear and distinct; even in confusion or dark or deafening noise, the private 'voice in the mouth' would continue to speak.

David Jones

Honeybees and 'yellow rain'

SIR—The misidentification as a biological warfare agent of the 'yellow rain' produced by defaecation during massed flights of the giant honeybee, *Apis dorsata*, in South-East Asia¹, has focused attention on the phenomenon of mass defaecation. We now suggest it can be explained by the need to prevent the temperature of bees' nests becoming so hot as to endanger the larvae.

When honeybees remain close together in the hot and humid tropics, their corporately generated heat must be dissipated. European honeybees (*A. mellifera*) evaporate water to keep the hive's temperature below about 37 °C, the thermal limit for larval life^{2,3}. Individuals of *A. mellifera* flying in extreme heat regurgitate nectar, let it evaporate on their mouthparts, and re-ingest the cooled droplet⁴ (a cycle we call gobbetting). *Apis dorsata* does the same both in the colony and in flight, maintaining a brood-rearing temperature of about 33.5 °C (ref. 5).

In tropical forests of Asia, *A. dorsata* nests in colonies of about 40,000 bees on large, single combs suspended in the open from high, breezy supports^{6,7}. Some aspects of their thermoregulatory behaviour are well known^{5,8}. At 26 °C, the midpoint of the daily ambient temperature, the temperature of the thorax in individual bees in a colony is at least 4.6 °C above that of the ambient air. The temperature excess (T_E) of the thorax at ambient temperatures above or below 26 °C is more than 4.6 °C, a result of thermoregulatory activity for cooling and warming, respectively.

One striking behaviour of *A. dorsata* is mass flight from the broodright colony, in which at least half of a colony's bees fly in gentle arcs up to about 20 m from the comb 5–6 min before returning. We have observed 19 such flights. In each case, the flying bees defaecated, a behaviour resembling cleansing flights in *A. mellifera*. We found 65–100 % of 306 bees in six colonies had faeces before mass flight, but only 0–44 % did so afterwards. The average volume of faeces dumped was 18.3 ± 3.2 μ l, weighing 24.6 ± 2.8 mg or 20 % of the total body weight. The mass specific heat of the faeces was 1.85 cal per g °C. With the minimum abdominal T_E of relatively inactive curtain bees of 2.52 ± 0.99 °C, a typical colony would dump 1,850 cal during mass defaecatory flights at ambient temperatures between 28 and 31 °C (ref. 5).

Apart from physical loss of heat in mass flight, reduction of a bee's body mass greatly increases thermoregulatory efficiency. Given a heat coefficient of 0.83 cal per g °C for animal tissue, the amount of energy needed to be lost to reduce the T_E of 5.1 ± 1.1 °C before mass flight to 3.4 ± 1.0 °C afterwards would be 0.22 cal for a

bee which has not defaecated (mean weight 123 mg), but only 0.14 cal for one which had (mean weight 98 mg). That is an energy saving of 36 % per participating bee and at least 15 % for the colony. The brood's T_E before and after mass flight was not significantly different.

Mass flight and defaecation occur when thermal stress is greatest. Of recorded mass flights, 60 % occurred between 0900 and 1200 when temperatures rise most rapidly, and another 20 % between 1600 and 1800, when it is often windless. For 33 flights where wind was recorded, 29 occurred in windless periods. The average ambient temperature during 17 mass flights was 29.5 ± 1.0 °C, with a relative humidity of 53 ± 6 %. Gobbetting always occurred before and after mass flights, indicating the continuing need for cooling. The number of gobbetting bees increases as the ambient temperature rises, whether or not mass flight occurs, and gobbetting bees always have lower T_E than do non-gobbetting ones⁵.

At air temperatures above 29 °C the

bees' T_E rises to and above 36 °C (ref. 5). Under still, humid conditions when heat is not easily lost to the air, temperatures in the curtain and brood rise above toleration (about 37 °C)⁵. Thus, activities which increase efficiency of heat loss, such as mass defaecation, can help avoid the nest temperature reaching a lethal temperature (about 37 °C) under still humid conditions when heat is not easily lost to the air and when the ambient temperature is high.

MAKHOZIR MARDAN

Plant Protection Department,
Universiti Pertanian Malaysia,
Serdang, Selangor, Malaysia 43400

PETER G. KEVAN

Department of Environmental Biology,
University of Guelph,
Guelph, Ontario, Canada N1G 2W1

1. Seeley, T.D., Nowicke, J.W., Meselson, M., Guillemin, J. & Akrotankul, P.A. *Scient. Am.* **253**, 128–137 (1985).
2. Seeley, T.D. & Heinrich, B. in *Insect Thermoregulation* 160–234 (Wiley, New York, 1981).
3. Free, J.B. & Spencer-Booth, Y. *Ent. exp. appl.* **5**, 249–254 (1962).
4. Heinrich, B. *Science* **205**, 1269–1271 (1979).
5. Mardan, M. thesis, Univ. Guelph (1989).
6. Morse, R.A. & Laigo, F.M. *Apis dorsata* in the Philippines (Monogr. Phil. Assoc. Entomol., 1961).
7. Deodkar, G.B. et al. *Indian Bee J.* **39**, 1–12 (1977).
8. Dyer, F.C. & Seeley, T.D. *J. exp. Biol.* **127**, 1–26 (1987).

Ceramics and flocculation

SIR—Kendall *et al.*¹ convincingly demonstrate the importance of particle clusters in limiting the strength of ceramics, but their analysis of the clustering process is incorrect.

The application of nucleation concepts to particle aggregation is open to serious doubt. The external surface energy of a cluster of particles must be less than the total surface energy of the individual particles that make up the cluster, because of particle–particle contacts. This effect would favour cluster formation rather than oppose it, as implied by equation (2) of Kendall *et al.*¹. In nucleation from solution, the formation of new crystal surface causes an increase in surface energy, which is outweighed by cohesive energy only for nuclei larger than a certain critical size.

Even if the analysis leading to their equation (4) is accepted as correct for clusters, the stationary value is not a dominant cluster size, but a critical size corresponding to a maximum of free energy. Clusters smaller than this size would be unstable, whereas for larger clusters, continued growth is favoured; there should be no dominant size. In practice, shear conditions will impose an upper limit on cluster size, but this aspect does not enter the treatment of Kendall *et al.*

With this conceptual error removed, there is no longer the apparent paradox that weaker attraction leads to larger aggregates, nor do the speculations con-

cerning biological growth have any foundation. The common-sense view that strong inter-particle attraction leads to large aggregates is still essentially correct.

JOHN GREGORY

Department of Civil Engineering,
University College London,
Gower Street,
London WC1E 6BT, UK

KENDALL REPLIES—We did not claim a formal analogy between nucleation theory and growth of particle clusters. There cannot be a formal analogy because particle aggregates can exhibit a large range of packing and structure whereas crystals are generally of uniform structure.

Our experiments, however, showed that flocculating a suspension of fine particles causes agglomerates to grow, somewhat like crystal growth, whereas vigorous agitation of the suspensions causes the aggregates to break down, unlike crystal growth. Therefore it seemed reasonable to conceive of a balance of growth against breakdown such that clusters of a particular size were produced under the influence of brownian motion.

Such a balance was obtained by considering the free energy of an agglomerate. The outer surface has a positive free energy and so tends to shrink, whereas the contact points within the agglomerate have a negative free energy and so tend to promote growth. The balance between these processes was found by seeking the stationary value of the sum of these posi-

tive and negative free-energy contributions for a perfect agglomerate. Agglomerates of this 'critical' size should neither grow nor shrink, and it is therefore plausible that such agglomerates should be found to predominate over those both bigger and smaller, which should be changing in size. However, as Gregory points out, this is a maximum of free energy and does not correspond to a stable equilibrium configuration, unless there is some as yet undiscovered perturbation that endows stability, as in the argon-clustering experiments of Hoare *et al.*².

Gregory states that "the common-sense view [is] that strong inter-particle attraction leads to large aggregates". Such a view is misguided. It is known that weak inter-particle forces lead to large, well structured aggregates. Many experiments have shown that uniform polystyrene spheres, when weakly flocculated, grow into close-packed domains of a few millimetres in diameter displaying opalescence, whereas strong flocculation causes the polystyrene to form small disordered aggregates which do not show opalescence.

K. KENDALL

ICI Advanced Materials,
PO Box 11,
Runcorn,
Cheshire WA7 4QE, UK

1. Kendall, K., Alford, N. McN., Clegg, W.J. & Birchall, J.D. *Nature* **339**, 130–132 (1989).
2. Hoare, M.R. *et al.* *J. Colloid Interface Sci.* **75**, 126–137 (1980).

Stereo suggestion

SIR—Tucker¹ and Wilson² suggest how publishers might alleviate one drawback to 'direct viewing' of stereo-pairs. But, even with years of practice (I acquired the technique unwittingly as a child by staring at repetitive wallpaper patterns), it requires an effort to achieve and maintain the stereoscopic view, and the depth of perception is never so clear as when a stereoscope is used.

Stereoscopes, however, are not easily obtained or cheap, and are too bulky to carry everywhere. I have recently found that a good substitute for a conventional stereoscope is to view the stereo-pair through two flat plastic Fresnel lenses, of the type which are now sold widely as magnifiers for reading. These lenses are not expensive, and two together have the shape and size of a credit card. The optical quality is surprisingly high, and the stereoscopic images are at least as good as those produced by the common folding stereoscope with moulded plastic bi-convex lenses.

ANDREW COULSON

Department of Molecular Biology,
University of Edinburgh,
Edinburgh EH9 3JR, UK

1. Tucker, V. *Nature* **337**, 605 (1989).
2. Wilson, J.H. *Nature* **339**, 433 (1989).

Size of aquatic endotherms

SIR—Downhower and Blumer¹ derive a lower size limit of 'aquatic endotherms' of 6.8 kg. This conclusion fails to account for the survival of smaller endotherms, such as adult little blue penguins (*Eudyptula minor* ~1.0 kg)² and newborn sea otters (*Enhydra lutris*, 2.0 kg)³, which spend considerable time in water.

In calculating the lower limit of body size in aquatic endotherms, Downhower and Blumer implicitly assume that the rate of heat loss by an endotherm is controlled by rate of convective heat loss from its surface. Furthermore, their model assumes that when an endothermic homeotherm enters water, the temperature difference between its surface and the environment remains the same as in air.

These assumptions are not valid for aquatic endotherms, most of which are surrounded by a layer of insulating blubber, fur or feathers that can reduce conductive heat loss so that surface temperature can be maintained close to the environmental temperature. Clearly, the rate of heat loss from the surface by convection in water is controlled by the rate of conduction from the animal's core, through its insulating layer, to the surface. Consistently, the rate of heat loss by aquatic endotherms in water is only 1.6–4.5 times greater than in air^{3–5}, not 90.8 times, as suggested by Downhower and Blumer.

When realistic values of heat production (metabolic rate), thermal conductance and temperature differences between the core and the aquatic environment are used in appropriate calculations, predicted minimum sizes of aquatic endotherms are much less than the 6.8 kg suggested by Downhower and Blumer, as the empirical evidence requires. Of course, factors other than thermoregulation may limit the sizes of endotherms encountered in nature.

S. INNES

D. M. LAVIGNE

Department of Zoology,
University of Guelph,
Guelph, Ontario, Canada N1G 2W1

DOWNHOWER AND BLUMER REPLY—In calculating the lower limit of body size in aquatic endotherms¹, we adopted the view that the same considerations for the lower limit of body size in terrestrial endotherms⁶ would hold for aquatic ones. Aquatic endotherms should be larger than terrestrial endotherms because rates of heat loss are greater in water than in air. We estimated the magnitude of that difference by comparing the convective heat transfer coefficient in air with that in water, holding size, temperature differentials and fluid velocity constant⁷. We coupled that difference with Pearson's estimate for the lower limit on body size in terrestrial

endotherms⁶, and an allometric equation relating conductive heat loss to body size (in terrestrial endotherms) to estimate the minimum size of a terrestrial endotherm that would remain a competent endotherm in water. Because cetaceans satisfy our requirement of being obligately restricted to water, we compared calculated lower limit of body size for aquatic endotherms with known weights of the smallest cetacean neonates.

Innes and Lavigne suggest there are smaller aquatic endotherms than those we considered, by equating the term 'aquatic endotherm' with any endotherm that spends time in water, rather than restricting it, to competent endotherms obligately restricted to a life in water. Thus, we do not include water shrews (8–18 g)⁵, little blue penguins³ (1–2 kg), harbour seals⁴ (22–34 kg, larger than our lower limit), or sea otters² (neonates, 2 kg).

Water shrews remain immersed for less than 60 s, dry their wetted fur by grooming, and drop their body temperature by 1–2 °C per 30 s during immersion⁵. In measuring conductive heat loss in little blue penguins, Stahel and Nicol³ say such measures may not adequately reflect the difficulties of thermoregulation in water: "given the same surface temperature in air and water penguins must withstand a rate of convective heat transfer that is two or three orders of magnitude greater in water than in air (Mitchell, 1974)". Although penguins and seals breed on land, sea otters do give birth in water, and spend much of their lives in water. But their young rest (out of water) on their mother², and are weaned at weights greater than 8 kg (J. Estes, personal communication). Their weight at weaning exceeds our predicted lower limit for body size.

Finally, Innes and Lavigne suggest that "realistic values . . . in appropriate calculations" would predict a lower minimum size for aquatic endotherms than we have calculated. But this requires that we ignore the increasingly important roles played by behaviour⁴, elevated metabolic rates³, limited times of immersion^{2,5}, degree of immersion² and hypothermia⁵ that are correlated with the severe thermoregulatory complications of invasion of aquatic habitats by endotherms smaller than our calculated lower limit.

J. F. DOWNHOWER

Department of Zoology,
The Ohio State University,
Columbus, Ohio 43210, USA

LAWRENCE S. BLUMER

Department of Biology, Kenyon College,
Gambier, Ohio 43022, USA

1. Downhower, J.F. & Blumer, L.S. *Nature* **335**, 675 (1988).
2. Kenyon, K.W. *The Sea Otter in the Eastern Pacific Ocean* (Dover, New York, 1975).
3. Stahel, C. & Nicol, S.J. *comp. Physiol.* **148B**, 93 (1982).
4. Hart, J.S. & Irving, L. *Can. J. Zool.* **37**, 447–457 (1959).
5. Calder, W.A. *Comp. Biochem. Physiol.* **30**, 1075 (1969).
6. Pearson, O.P. *Science* **108**, 44 (1948).
7. Mitchell, D. in *Environmental Physiology* (ed. Robertshaw, D.) 1–32 (Butterworths, London, 1974).

Physicist and philanderer

P. W. Atkins

Schrödinger: Life and Thought. By Walter Moore. Cambridge University Press: 1989. Pp.513. £25, \$39.50.

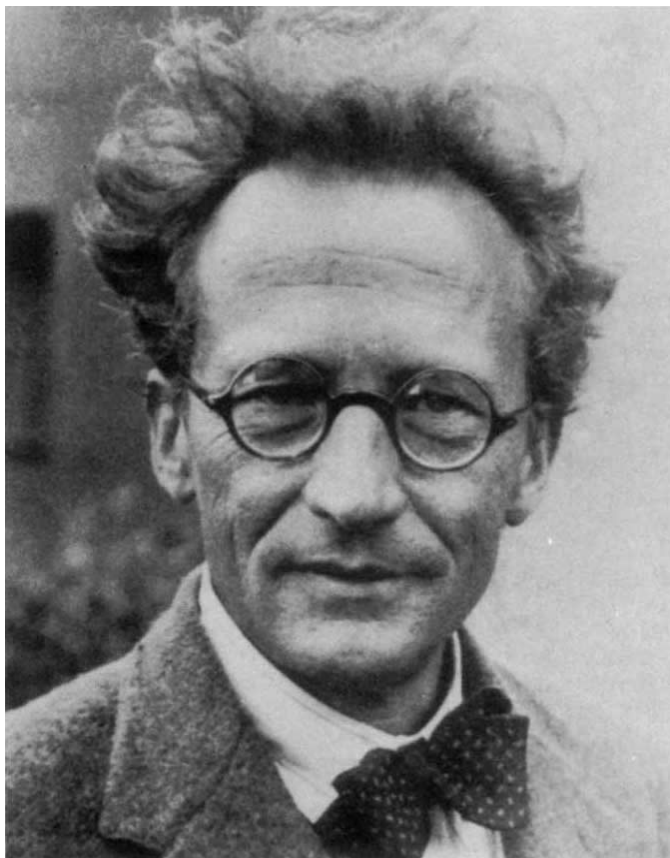
WHAT is life? That Erwin Schrödinger knew the answer, in more ways than one, is revealed to us in this biography. He suffered the deprivations of two world wars, and had the euphoria of the award of a Nobel prize; he enjoyed a *ménage* of at least *trois*, captivated and hunted young women, and through his intelligence and charm captured them. He felt himself to be almost a component of the cosmic being, and that the world was his oyster. But he was flawed in conventional earthly terms, for his behaviour, charitably interpretable as naïveté, could be cowardly, hurtful and ungrateful. He is fortunate to have the admiring biographer that Walter Moore has proved to be.

Schrödinger arrived in the world and left it in Austria, in 1887 and 1961 respectively, and we are not long gone from his centenary year. He was born into the political turmoil that repeatedly slashed the face of Europe, but as if in compensation enjoyed the comparable but happily more abstract turmoil that was the mark of physics over the same period. His life spanned the discovery and maturing of modern physics, and he contributed notably to a formulation of quantum mechanics that is still the more widely used and which is normally encountered first.

For a summary of a life in a nutshell — and in many respects Schrödinger's life was bigger than any familiar nut — it is enough to remark that he grew up among the classical physicists of Vienna, endured the deprivations of that city in the First World War, and went on to propose his wave equation in an outburst of creative energy in the mid-1920s while holding a chair in Zürich. After fittingly succeeding Max Planck in Berlin in 1927, he (a Protestant) escaped to Oxford in 1933. But he foolishly returned to the heart of Nazi-raddled Graz, only to find it necessary to leave a year or so later for Oxford again and later Ghent. Meanwhile he had been pursued by De Valera, whose ambitious and admirable scheme to found an Institute for Advanced Studies in Dublin came to fruition despite the privations to which Eire was subject. Schrödinger was appointed its first director; there he stayed from

1940 to 1956 in one capacity or another, but in the end was drawn back to his motherland, to die in the Tyrol.

Such was Schrödinger's public *vita*, the spring of his awards, the Nobel prize in 1933, and his immortality in physics. Yet there was a private side that occupies much of this biography. The private side



Erwin Schrödinger — "literally felt himself at one with God".

sparkles from the jacket photograph and from the more permanent collection of images within. Here we see not the mathematically sophisticated but sometimes physically misled creator of new worlds and extender of the domain of physics into the realm of life, but the urbane, handsome, witty, wooer of women of all ages, the younger the better. Nymphets, the young unattached, and wives all came within his questing sexuality, and more than one emerged impregnated as the mother of his child. His occasionally unrequited quest for love (or, at least, active loving) began before his main work was done. But with the confidence that came with success, fame spurred him to a greater exuberance, and in discarding conventional morality he

also discarded sombre jackets for Tyrolean suits, much to the perturbation (on both counts) of those of stiffer frames of mind. All this was done against the background of his stolid wife Anny's active complaisance. It is fitting that in the days before his death he appears to have recognized the freedom and — in her own way — the love and faithfulness that she brought to their unconventional marriage.

Yet, skilled mathematician, lucky physicist and engaging philanderer that he was, there was also a darker, more unpleasant and (to the partly informed at least) reprehensible side to Schrödinger's demeanour and activities. Professional and sexual success had gone to his head, and in some respects Schrödinger considered himself to be God. Women were his admiring angels, and only rarely was his self-felt superiority brought into question by some who could cut him down to size. With his Vedantic view of the cosmos he literally felt himself at one with God, and presumably felt himself a particularly witty protuberance into reality of an underlying unity. Hubris, or just plain thoughtlessness, led to ill treatment of those who had helped him. Lindemann, who had done so much to induce ICI to provide funds to support Schrödinger in England, was a particular, probably unconscious, target of this kind of abuse and ingratitude. Others suffered too, perhaps Anny most of all.

Schrödinger also appears to have been a coward, and I do not think that the aura of naïveté that is widely claimed for him (though not everywhere in this quite well-balanced book) is enough to excuse his famous grovelling letter written in Graz in 1938:

In the midst of the exultant joy which is pervading our country, there also stand today those who indeed partake fully in this joy, but not without deep shame. . . . I make this confession willingly and joyfully. I believe it is spoken from the hearts of many, and hope thereby to serve my homeland.

The newspaper that published the letter leapt on the support it most definitely appears to give for the Nazis. His excuse was the need to remain free, and hence the concomitant need to resort to duplicity. That may be so, for Schrödinger was morally shallow. The letter pleased few, however, and he lost Einstein's respect. That loss was a severe sadness, for Schrödinger was pleased that Einstein, his earthly hero, had regarded him as an intellectual brother as the two struggled vainly for a unified field theory after their powers had fallen from their prime.

I find it difficult to judge whether Schrödinger was truly great — as a scientist; as a man he was no conventional saint. I suspect that he was a great man of the second division. In his early work his mathematical skill was alloyed with a baser metal, and the contaminant did not allow him to judge the physical plausibility of everything he did. He was perfectly in his element, mathematical manipulation, when dealing with his wave equation, and the sunburst of solutions that accompanied its first publication. Yet that moment in his life had no precedent and was not to be repeated, despite some notable contributions to relativity. He was certainly farsighted in his pushing for physics to be extended to form the foundations of biology, but his inclinations to the tenets of Eastern religions (as far as an orthodox scientist can judge) acted as a

cataract to dim his vision. He also wrote poetry (particularly to pull in a harpoon that had been let slip to achieve an amorous conquest); again, as far as I can judge from the numerous examples in the text, he was not a poet.

Physics is certainly the better for Schrödinger's existence, and that is the essence of true immortality. Dublin was also undoubtedly intellectually enriched by his lengthy sojourn there and his early moulding of its famous Institute, and that is a valuable contribution to civilization. Some friends, I suspect, have been left not a little poorer; but in the great accounting, perhaps friends don't count, especially if you think you are God Almighty. After all, what is life? □

P.W. Atkins is in the Physical Chemistry Laboratory, University of Oxford, and is a Fellow of Lincoln College, Oxford OX1 3DR, UK.

Light work

O. S. Heavens

The Fabry-Perot Interferometer: History, Theory, Practice and Applications. By J. M. Vaughan. Adam Hilger: 1989. Pp. 583. £60, \$130.

UNTIL the arrival of the laser, optics tended to be dismissed as one of the bits of physics that was essentially completely dealt with. James Clerk Maxwell had after all given a comprehensive and fundamental description of light — a mere slice of the broader spread of the electromagnetic spectrum — which accounted elegantly for all the known phenomena such as propagation, interference, diffraction and scattering. Although the need to recognize the quantum character of light (and the wave character of matter) somewhat disturbed the serenity, there seemed no need to revise the essentials of Maxwell's ideas, nor to expect any dramatic developments.

Nobel prizes flowed steadily in the direction of particle physicists — until 1964. In that year the award of the prize to Townes, Prokhorov and Basov signalled the return of optics to the frontier region. Following the notions outlined by Einstein in 1917, which were exploited in the microwave region of the spectrum in 1954 (maser), the laser appeared in 1960 and rapidly transformed most areas of optics. Within a few years, many of the quantities associated with 'ordinary' light sources had changed by many orders of magnitude, especially intensity, brightness and monochromaticity. By now there are few disciplines in the sciences and engineering that have not been profoundly affected.

Both the operation of the laser and many of its remarkable applications are based on the Fabry-Perot interferometer,

a deceptively simple device consisting of two flat, partly silvered glass plates rigidly held parallel to one another. First described by Perot and Fabry in 1899, the device immediately made it possible to determine the wavelength of spectral lines to an unprecedented accuracy — better than one part in a million. The historical background provided in this book leaves one fascinated and full of admiration for the thoroughness and ingenuity of the experimenters at the turn of the century.

The step to the laser was in a sense very simple. Take a pair of Fabry-Perot plates, pull them apart, and place a discharge tube (say a neon sign) in between them. (This was in fact tried by Tolansky in 1956 — not to try to make a laser but to "see what happened". Nothing did.) When things are properly adjusted an intense light beam, less than a millimetre in diameter, streams out of the system. The apparent simplicity belies the great usefulness of a light beam which has very regular oscillations (coherence), is of high intensity and has an extremely well-defined wavelength (colour).

Dr Vaughan gives many examples of the dramatic contributions which the laser, in conjunction with the Fabry-Perot interferometer, has made to the fields of atomic spectroscopy, astronomy, astrophysics, light-scattering, metrology, infrared detection and many others. In fact, the content of the book is much broader than the title suggests, in that the 70 or so pages of appendix bring the reader thoroughly up to date with the whole range of the many associated techniques needed to exploit this remarkable device to the full. The book will be enjoyed at many levels — for its historical interest, as an overall perspective of some 25 years of exciting developments and as a valuable source of information on the state of the art. □

O. S. Heavens is in the Department of Physics, University of York, York YO1 5DD, UK.

Opiates for the people

Peter Newmark

Brainstorming: The Science and Politics of Opiate Research. By Solomon H. Snyder. Harvard University Press: 1989. Pp. 208. \$22.50. To be published in Britain next month, £17.95.

PRESIDENT Bush's administration has just declared war on drugs, and wants to spend an extra two billion dollars next year in controlling the problem. Vast sums will go into law enforcement, prison space, education, workplace inspections and media campaigns. Half a billion dollars in all will be set aside to fight the battle against the trafficking barons in South America and in providing protection for the legal officers who stick to their unenviable jobs. The same amount will be spent on increasing and improving drug treatment, which is particularly poor for cocaine users.

How much extra is to be spent on biomedical research, which might lead to completely new approaches to the prevention and treatment of drug addiction? Nothing, as far as can be told. Could it be that the amount is so little as not to warrant a mention? Or that the swash-buckling image is so important to those who formulate strategy that the scientists simply did not stand a chance? Or is it that the President was given a pre-publication copy of Solomon Snyder's thoughtful new book, but gave it too narrow a reading?

It was Richard Nixon's war on drugs, launched in 1971 to counter an epidemic of heroin addiction, that also launched Snyder into opiate research. At that time, he "hardly knew heroin from horse-radish" but did know Jerome Jaffe, the man that Nixon put in charge of an office created to coordinate drug-abuse programmes. Jaffe and Snyder were both trained as psychiatrists in the 1960s and had become interested in the biological approach to mental illness, so Snyder's suggestion that some money should be spent on drug research centres received a sympathetic hearing. But such was the opposition to spending on research rather than treatment, that it was many months before Snyder found himself applying for a share of the two million dollars to be spread among six research centres.

Snyder was convinced that his first goal should be to establish the site of action of opiates and to identify the cell-surface receptor through which he was sure they acted. But suspecting that such a project would be thought too bold by his peers, he adopted the well-known ploy of applying for funds to carry out research on

amphetamines that he had already more or less completed, and then, having obtained funds, switching tracks. By summer 1972, Snyder had set Candace Pert, a self-confident graduate student, the task of identifying the opiate receptor.

Nine months later, to coincide with publication of the paper that told of their success, Snyder found himself facing the world's press. The reason was this. Snyder's funds came from a specific political initiative and the White House — as its counterparts in most places at most times — tended to the view that nothing of value comes quickly enough out of basic research to reflect well upon those who have invested in it. Sensing that the discovery of the opiate receptor was an exception, the director of drug-abuse research at the National Institutes of Health set up a press conference.

The discovery indeed led to vastly simplified tests for new agonists and antagonists of the receptor, that might lead to improved treatments for heroin addiction. It also enabled the distribution of the receptor in the brain to be mapped to the pain pathways. And when, in 1975, Hans Kosterlitz and John Hughes in Aberdeen became the first to characterize some of the peptides that are natural ligands for the receptor, and so the body's own painkillers, the way seemed set for pharmaceutical companies to produce a new generation of analgesics. Based on the natural compounds, which were surely not addictive, they would replace morphine and its relatives.

There was a catch, however. It emerged

that the only reason that the natural ligands for the opiate receptor are not addictive is that they are very short-lived. For therapeutic purposes, the pharmaceutical companies had to produce long-lasting analogues. These proved to be addictive and have never reached the market. Thus a narrow reading of Snyder's story might confirm the view that pouring money into basic research seldom yields immediate returns of the kind that would satisfy politicians.

Snyder writes well. He is adept at explaining technicalities and is forthcoming about his own motivation and shortcomings. He confesses that his research has often been influenced as much by the need to stake out territory, establish priorities and defend reputations as by any noble notion of furthering the cause of science, and he admits to being a colossal klutz when it comes to experiments. Consequently, he never does them himself, instead leaving them to his ten or so graduates and postdocs, none of them permanent. Obviously caring about the well-being of his students, he nevertheless cannot be easy to work for.

In 1978, the Lasker Awards, awarded annually in the biomedical sciences, were given to Snyder, Kosterlitz and Hughes. Not surprisingly, Pert was less than pleased by her exclusion and was not reluctant to say so. One would have expected the ambitious Snyder to mention the prize, and the psychiatrist Snyder to explore the circumstances. Strangely, there is no word from either. □

Peter Newmark is Deputy Editor of *Nature*.

Giving reasons

Alan Holland

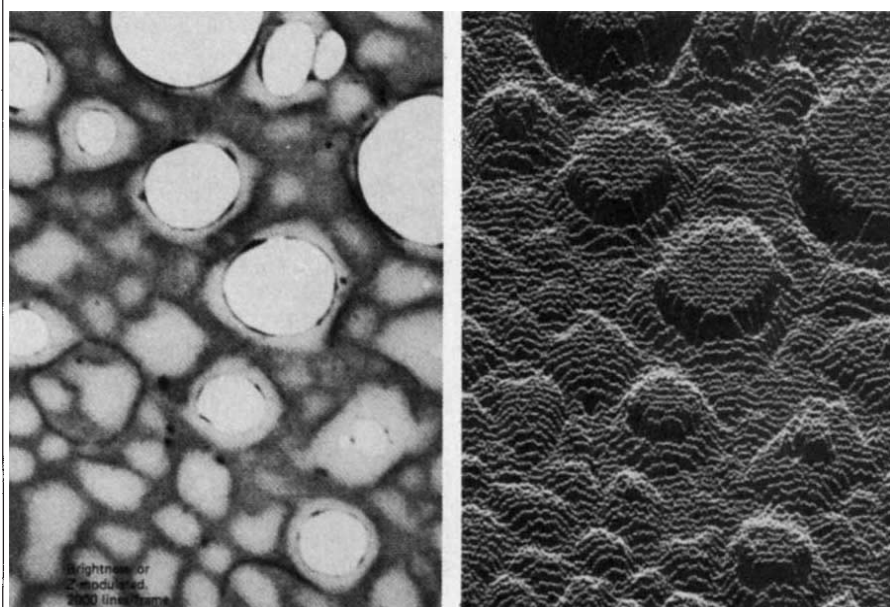
Animal Consciousness. By Daisie Radner and Michael Radner. *Prometheus*: 1989. Pp. 253. \$39.95. To be published in Britain next Spring, £29.95

CONSCIOUSNESS, it seems, is an embarrassment to scientists and philosophers alike. The neurologist R. J. Joynt declares that he has been trained not to deal with it. The philosopher Patricia Churchland would have it as on the way to obsolescence.

Animal consciousness, then, is an embarrassment on legs. For it lacks even the accreditation of first-person testimony. In the latest of a small crop of books on the subject which have appeared this year, Daisie and Michael Radner stand up for animal consciousness as an aspect of animal life which any thoroughgoing evolutionary ethology should recognize. Philosophers themselves, the Radners have done their scientific homework and can be read with profit by anyone involved in the study of animal natures. Their purpose is to uphold the legitimacy of both scientific and philosophical study of animal consciousness, by clearing away some of the stumbling blocks to that study.

Chief among the stumbling blocks is cartesian metaphysics, which sees human beings as "composed of a soul and a body". In itself the human body is but a "machine made of earth", and animals are assigned the same status as human bodies, lacking a mind or soul. In the first half of the book — "The Breakdown of the Beast-Machine" — the Radners show how the resources of the cartesian tradition, even granting all its main assumptions, fail to disprove animal consciousness. Contrary to what Descartes maintained, the actions of some animals can be construed, and in some cases perhaps can only be construed, as employing reason — provided only that some middle ground is recognized between perfect reason and no reason at all. Sensation and the powers of communication are denied to the animals only on the unfounded and exclusive presumption that they have to involve pure thought. Of particular interest is the Radners' discerning of a dual notion of consciousness in Descartes — roughly, a reflective and a non-reflective awareness — which is actually conducive to the recognition of animal consciousness rather than the reverse. Their reading of Descartes is plausible and they are no doubt right to suggest that received accounts of his concept of mind are distorted by the undue attention that has been paid to the rather special preoccupations of the *Meditations*.

The Radners claim that, in the light of the theory of evolution, the existence of human consciousness makes the existence



Highlighting takes on a new meaning when special image-processing techniques are applied to electron micrographs. On the left is a standard micrograph (brightness, or Z, modulated) of a defective carbon film: perforations and thin patches are brighter than the surrounding film. On the right, the intensity information is used to modulate the vertical deflection of the left-to-right raster lines (Y-modulation), so that the eye sees it as topography on a surface. These images are taken from the new, amended paperback edition of *The Principles and Practice of Electron Microscopy* by Ian Watt, published by Cambridge University Press.

of animal consciousness probable. If they are correct, then the persistence of the cartesian legacy in the period after Darwin — the subject of the book's second half — is all the more surprising. Perhaps their claim is overstated. Arguing from the case of one species seems mighty risky. And perhaps the significance of consciousness is magnified in human eyes so that we wrongly think it must be expected to exist in many other animal forms. The authors' preferred explanation points to the failure — for example, of the early comparative psychologists — to find an *explanatory* role for consciousness, a place in the causal network. To this they add the positivist-inspired doubts about the testability of ascriptions of conscious states, and some powerful reworking of Descartes' doubts about the capacities of animals for communication and reasoned action. The Radners confront these new and renovated problems with resolution and ingenuity. Their re-evaluations of three prominent examples of alleged mindlessness in animals are among the best things in the book which, overall, is well constructed and adroitly argued.

My chief criticism concerns what they haven't done, or haven't done thoroughly enough. Having argued that 'there is something it is like to be an animal' — to use Tom Nagel's illuminating phrase — the next question is how we are to discover *what* it is like. Although they recognize the problem, the Radners don't take us very far along this road. In the matter of

whether it is better to be a dissatisfied human being or a satisfied pig, John Stuart Mill was confident that human beings know both sides of the question.

Although they must agree with Mill in principle, the Radners rightly observe that matters are not that simple. Because desires are integrated differently in a pig, they say, and because pigs have "desires of their own" (they don't say how they know this), the comparative judgement is problematic.

In another case involving attempts to communicate with primates, they observe, reasonably enough, that the question asked of the gorilla, Koko, "Where do gorillas go when they die?" is a human question. For Nim the chimpanzee, who had tested new teachers by aggressive displays, they suggest "Me boss you?" as a more appropriate reflection of Nim's world. Yet, it seems to me, they may be seriously underrating the difficulties of entering the porcine or simian worlds. It is within the human world that Nim's response is construed as aggressive. It seems rash to assume that the response bears that construction in the rather differently structured world of the chimpanzee. In problems such as these lie the fascination and challenge of the study of animal consciousness which, in a suitably sensitive and disengaged form, seems barely to have begun. □

Alan Holland is in the Department of Philosophy, University of Lancaster, Lancaster LA1 4YT, UK.

the idea arose and was systematically and rapidly developed. In this book, the Cetus group and their co-authors are extremely successful in conveying both the theoretical and the applied aspects of PCR.

An initial section on basic methodology and its application provides a clear yet detailed chapter describing protocols and conditions for PCR and an excellent chapter on the polymerase enzyme obtained from *Thermus aquaticus* (Taq). Through its stability at high temperatures, Taq has permitted the technique to be automated and hence widely applied. Although Cetus has a substantial commercial interest in both the enzyme and a PCR thermal cycler, there is little direct promotion of their products — except perhaps for the criticism of water-cooled thermal cyclers because they waste water and are therefore ecologically unsound!

The second part of the book is devoted to evaluating applications. Modifying DNA, cloning new genes, detecting mutations, inverted PCR and numerous other variations on the basic technique are described. Not all of the modern adaptations are included — anchor PCR, quantitation (and its problems) and CA repeat mapping are not discussed. But that was inevitable; not only are things moving so fast, but a comprehensive review of research applications would have made the book unmanageable.

The field where PCR is most likely to have its greatest impact is human genetics, and the contributors to the third section, "Medical Applications", are mostly drawn from this camp. As well as chapters on genetic diagnosis, HLA polymorphism and oncogenes, there are discussions of forensic applications and detection of infectious disease. These latter two chapters are likely to be of special importance in the future.

A notable feature of the book is its emphasis on practical aspects of the technique — every chapter is written by innovative scientists and is accompanied by detailed protocols. In particular, careful attention is paid to contamination. This failing of PCR is related to its greatest strength, because extreme sensitivity commonly leads to the spurious amplification of DNA contaminants. The problem is thoroughly addressed by the authors, and if their advice is taken it can clearly be contained.

This volume is long overdue. It lives up to all expectations, and will be valuable to both the neophyte and the experienced PCR scientist. The only recommendation I can add is that it should be purchased and read quickly, for if the field continues to move as quickly as it has the authors will be writing the next edition early in 1990. □

John Bell is in the Molecular Immunology Group, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK.

Out of chains

John Bell

PCR Technology: Principles and Applications for DNA Amplification. Edited by Henry A. Erlich. Stockton, New York/Macmillan, London: 1989. Pp.246. Pbk \$19.95, £15.95.

COMPILING a book on a technique that is developing rapidly presents considerable difficulties. It is perhaps for this reason

that almost three years have passed without a serious attempt being made to assemble a volume on the polymerase chain reaction (PCR). This technique, which permits the *in vitro* amplification of specific DNA sequences, is now one of the most powerful tools available to molecular biologists. New applications and adaptations have been appearing so quickly that even journals are having a difficult time keeping up.

No group is more suited to review the field than Henry Erlich and his colleagues at Cetus. It was from their laboratory that

decision was taken to include the developments of 1988 and early 1989. Principal amongst these is PCR technology, the subject of a new chapter. Overall, the material has been split into three volumes, and although the authors remain the same the second edition becomes Sambrook *et al.* rather than Maniatis *et al.*

Sambrook *et al.* will face much more competition than they did before (for some of the contenders see *Nature* 333, 309; 1988). But the work's biblical status and its remarkable price (\$97) will no doubt make it a compulsory purchase. TL

PCR and more besides

'LONG-awaited' is the tag publishers on occasion apply to their books that appear months, even years, after they were first announced — and, it turns out, to indifferent public reaction.

That should not be the fate of the long-awaited second edition of *Molecular Cloning: A Laboratory Manual*, even though molecular biologists are still waiting for it. First announced for publication by Cold Spring Harbor Laboratory Press in October 1988, the second edition will appear next month.

The reason for the delay is that the

Inositol phosphates and cell signalling

Michael J. Berridge & Robin F. Irvine

Inositol 1,4,5-trisphosphate is a second messenger which regulates intracellular calcium both by mobilizing calcium from internal stores and, perhaps indirectly, by stimulating calcium entry. In these actions it may function with its phosphorylated metabolite, inositol 1,3,4,5-tetrakisphosphate. The subtlety of calcium regulation by inositol phosphates is emphasized by recent studies that have revealed oscillations in calcium concentration which are perhaps part of a frequency-encoded second-messenger system.

KNOWLEDGE of the phosphoinositides and their role in cell signalling has expanded enormously since we reviewed this field five years ago¹. Stimulation of cell-surface receptors initiates hydrolysis of a membrane-bound inositol lipid, which produces at least two second messengers—diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃). These messengers are generated by a membrane transduction process comprising three main components: a receptor, a coupling G protein and phosphoinositidase C (Figs 1 and 2). DAG acts by stimulating protein kinase C (ref. 2), whereas Ins(1,4,5)P₃ releases calcium from internal stores^{1,3}. These pathways form the cornerstone of an ubiquitous transduction mechanism now known to regulate a large array of cellular processes including metabolism, secretion, contraction, neural activity and cell proliferation¹⁻⁷. The second messenger role of DAG has been reviewed recently², and here we concentrate on the contribution of the inositol phosphates.

An enormous number of inositol phosphates have now been found in eukaryotic cells, and details of some of the metabolic pathways linking them have been elucidated¹⁰⁻¹² (summarized in Fig. 1; see panel on right for details of inositol phosphate numbering). A myriad of suspected or postulated functions have been ascribed to them; some of the more plausible are mentioned briefly below, but here we concentrate principally on the messenger functions of Ins(1,4,5)P₃ and related compounds, and particularly on their involvement in the generation of intracellular calcium signals. As more and more studies are made on single cells, it is becoming apparent that the phosphoinositide transduction system is highly organized in both space and time, and we present a spatiotemporal model that may help to unify the emerging ideas about intracellular calcium signalling.

Multiple calcium pools

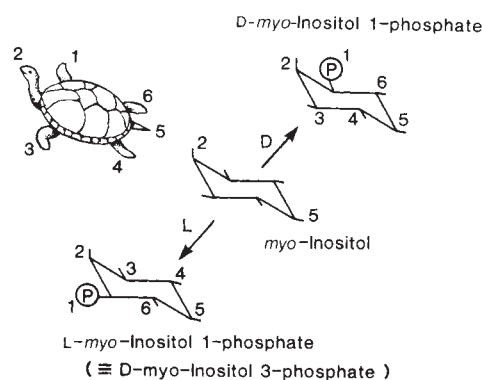
When cells respond to calcium-mobilizing agonists that act through the hydrolysis of inositol lipids, they draw upon both intracellular and extracellular sources of calcium. Although the latter remains constant, the size and distribution of the internal pools is highly variable. Ins(1,4,5)P₃ releases calcium from a non-mitochondrial pool which has characteristics which suggest that it is the endoplasmic reticulum (ER)¹³, but only part of this pool seems to be Ins(1,4,5)P₃-sensitive; on average Ins(1,4,5)P₃ releases approximately 30–50% of the calcium taken up by the non-mitochondrial pool of calcium, the remainder of which can be released by calcium ionophores. This evidence implies that the Ins(1,4,5)P₃-insensitive pool is a separate membrane compartment which may have distinct calcium-pumping characteristics¹⁴.

The anatomical location and identity of the Ins(1,4,5)P₃-sensitive and -insensitive pools are uncertain. Supattapone *et al.*¹⁵ have purified a protein of relative molecular mass 260,000 (260K) that is thought to be the Ins(1,4,5)P₃ receptor. Immunocytochemical studies using a specific antibody on Purkinje cells (which have an extraordinarily high receptor density) reveal that this receptor is localized on the nuclear envelope and on parts of the ER, particularly near the nucleus¹⁶. Another candidate for the Ins(1,4,5)P₃-sensitive calcium pool,

at least in peripheral tissues, is the calciosome, a small membrane vesicle which has some properties characteristic of the sarcoplasmic reticulum of muscle¹⁷. Calciosomes contain the calcium-binding protein calsequestrin and have calcium pumps with immunological properties resembling those in muscle. It is still not clear how calciosomes are related to the Ins(1,4,5)P₃-sensitive or -insensitive stores.

Stereochemistry and abbreviations of inositol phosphates

There has been much confusion in the literature concerning the nomenclature of the family of inositol phosphates. Strict adherence to the original IUPAC rules has added further confusion in that addition or removal of a phosphate could necessitate a switch between D- and L-systems of numbering. *myo*-Inositol, represented here in its chair conformation, has a plane of symmetry running through carbon atoms 2 and 5 thus dividing the molecule into chiral halves⁸. Agranoff's turtle is a useful mnemonic for remembering inositol phosphate numbering⁹. Substitution on carbon atoms 1,3,4 or 6 will alter this symmetry and the product must now be designated as D or L to define its position as illustrated for the D- and L-*myo*-inositol 1-phosphate enantiomers (which both occur naturally, see Fig. 1).



A new convention, recently published by the IUPAC, suggests that all inositol phosphates may be numbered as the D-*myo*-inositol derivative (abbreviated as Ins). Using this convention, the L-*myo*-inositol 1-phosphate (shown above) would adopt the numbers of the D-configuration and becomes D-*myo*-inositol 3-phosphate, abbreviated to Ins(3)P. The following are abbreviations for some of the more common inositol lipids and inositol polyphosphates appearing in the text:

Ins(4)P	D- <i>myo</i> -inositol 4-monophosphate
Ins(1,4)P ₂	D- <i>myo</i> -inositol 1,4-bisphosphate
Ins(1,4,5)P ₃	D- <i>myo</i> -inositol 1,4,5-trisphosphate
Ins(1:2cyc,4,5)P ₃	D- <i>myo</i> -inositol (1:2-cyclic,4,5)-trisphosphate
Ins(1,3,4,5)P ₄	D- <i>myo</i> -inositol 1,3,4,5-tetrakisphosphate
InsP ₅	Inositol pentakisphosphate (unspecified isomer)
InsP ₆	Inositol hexakisphosphate (phytic acid)
PtdIns	Phosphatidylinositol
PtdIns(4)P	Phosphatidylinositol 4-phosphate
PtdIns(4,5)P ₂	Phosphatidylinositol 4,5-bisphosphate

Another question concerns whether or not the $\text{Ins}(1,4,5)\text{P}_3$ -insensitive pool can contribute to calcium signals initiated by $\text{Ins}(1,4,5)\text{P}_3$. Studies on permeabilized cells and cell fractions have shown that GTP can release Ca^{2+} from both pools independently of $\text{Ins}(1,4,5)\text{P}_3$ (ref. 18) and that this release can be blocked by treatments that have no effect on the $\text{Ins}(1,4,5)\text{P}_3$ -

sensitive system. These properties closely resemble the GTP-dependent control of vesicular protein transport through the Golgi¹⁹ and, with the relatively constant levels of GTP in cells, the physiological significance of GTP-induced calcium release in intact cells is doubtful. It has been suggested, however, that GTP may somehow enlarge the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pool at

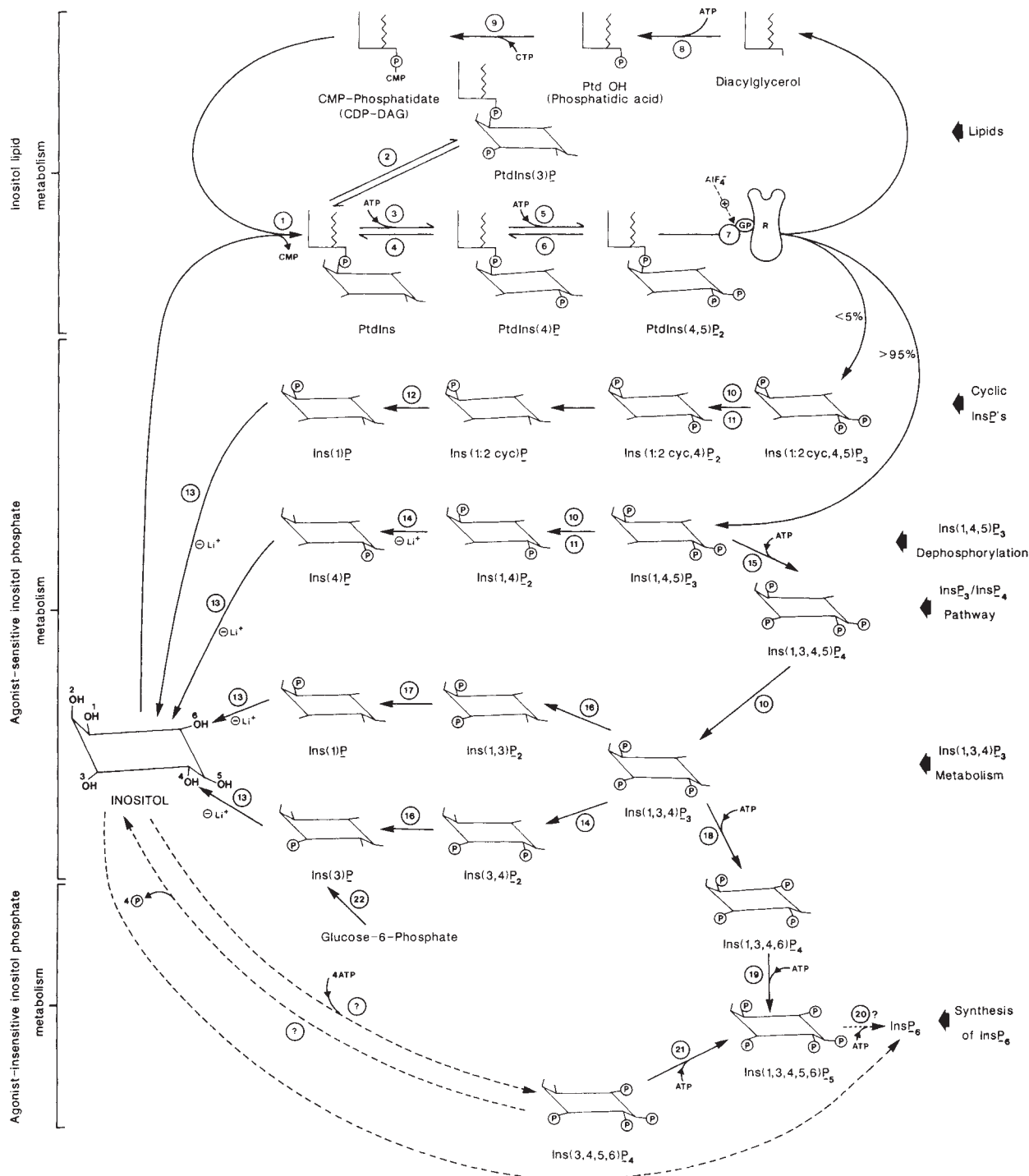


FIG. 1 Summary of known (solid arrows) and suspected (dashed arrows) routes of metabolism of compounds containing inositol and phosphate. To avoid further complexity, the phosphatidylinositol glycans are not illustrated. *myo*-Inositol is represented in its chair configuration and all the inositol phosphates are numbered in the D-isomer configuration (see panel on previous page). The enzymes are 1, PtdIns synthetase (CMP-PA; inositol phosphatidyltransferase); 2, PtdIns-3-kinase (type I); 3, PtdIns-4-kinase (type II); 4, PtdIns(4)P phosphomonoesterase; 5, PtdIns(4)P-5-kinase; 6, PtdIns(4,5)P₂ phosphomonoesterase; 7, phosphoinositidase C; 8, diacyl-

glycerol kinase; 9, CMP-PA synthetase; 10, $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ -5-phosphatase; 11, $\text{Ins}(1,4,5)\text{P}_3$ -5-phosphatase; 12, $\text{Ins}(1:2\text{cyc})\text{P}$ phosphodiesterase; 13, InsP phosphatase; 14, inositolpolyphosphate-1-phosphatase; 15, $\text{Ins}(1,4,5)\text{P}_3$ -3-kinase; 16, inositolpolyphosphate-4-phosphatase; 17, $\text{Ins}(1,3)\text{P}_2$ -3-phosphatase; 18, $\text{Ins}(1,3,4)\text{P}_3$ -6-kinase; 19, $\text{Ins}(1,3,4,6)\text{P}_4$ -5-kinase; 20, $\text{Ins}(1,3,4,5,6)\text{P}_5$ -2-kinase (probably does not exist, as $\text{Ins}(1,3,4,5,6)\text{P}_5$ is unlikely to be the precursor of InsP_6); 21, $\text{Ins}(3,4,5,6)\text{P}_4$ -1-kinase; 22, $\text{Ins}(3)\text{P}$ -synthetase.

the expense of the $\text{Ins}(1,4,5)\text{P}_3$ -insensitive pool²⁰ and this process may be controlled by $\text{Ins}(1,3,4,5)\text{P}_4$ (ref. 11). The $\text{Ins}(1,4,5)\text{P}_3$ -insensitive pool may also help to amplify and propagate the calcium signal derived from the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive store by a mechanism of calcium-induced calcium release (see below).

$\text{Ins}(1,4,5)\text{P}_3$ -induced calcium mobilization

To release calcium, $\text{Ins}(1,4,5)\text{P}_3$ must bind to receptors that are somehow linked to calcium channels connected with the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive calcium pool. A variety of tissues have a high-affinity stereospecific binding site for $\text{Ins}(1,4,5)\text{P}_3$, which recognizes D- $\text{Ins}(1,4,5)\text{P}_3$ much better than L- $\text{Ins}(1,4,5)\text{P}_3$ (refs 21, 22). This may be the receptor responsible for releasing calcium, because both processes have a similar profile of responses to InsP_3 derivatives and enantiomers^{3,21,23}. Heparin also inhibits both the binding of $\text{Ins}(1,4,5)\text{P}_3$ to its purified receptor¹⁵ and the mobilization of calcium from liver cells²⁴.

Individual $\text{Ins}(1,4,5)\text{P}_3$ -sensitive calcium channels (sensitive to heparin) with conductances of ~ 10 pico-Siemens (pS) have been recorded from membrane vesicles isolated from the sarcoplasmic reticulum of aortic smooth muscle, which have been incorporated into planar lipid bilayers²⁵. The action of $\text{Ins}(1,4,5)\text{P}_3$ seems highly cooperative, suggesting that three molecules are required to open the calcium channel²⁶. Using a caged molecule from which free $\text{Ins}(1,4,5)\text{P}_3$ can be released by a flash of laser light²⁷, a distinct threshold can be demonstrated above which the response seems to be all-or-nothing²⁸; paired light flashes produced facilitation²⁸. Overall, this cooperativity may help to explain the long latencies that exist when cells are stimulated with calcium-mobilizing agents²⁹. Finally, reconstitution experiments with the purified receptor suggest that the $\text{Ins}(1,4,5)\text{P}_3$ -binding site, and the Ca^{2+} channel which it controls, may reside in the same protein (C. D. Ferris, R. L. Haganir, S. Supattapone and S. H. Snyder, personal communication).

The $\text{Ins}(1,4,5)\text{P}_3$ -induced release of calcium can be modulated by a variety of physiological and pharmacological agents. Classical calcium-channel blockers have no effect^{30,31}, but release is inhibited by cinnarizine and flunarizine³⁰ and by the potassium channel blocker tetraethylammonium (TEA)³¹. In the absence of potassium, $\text{Ins}(1,4,5)\text{P}_3$ is incapable of releasing calcium but other monovalent cations ($\text{Na}^+ \gg \text{Tris}^+ > \text{Li}^+$) can replace potassium, which functions to neutralize the charge developed by the efflux of calcium³². In cerebellar membranes, both the binding of $\text{Ins}(1,4,5)\text{P}_3$ to its receptor and its calcium-

mobilizing action are sensitive to calcium^{15,33}. This effect seems to depend on a calcium-mediator protein (calmodin) which is particularly abundant in brain but sparse in peripheral tissues³⁴. Also, the $\text{Ins}(1,4,5)\text{P}_3$ receptor isolated from brain can be phosphorylated by cyclic AMP-dependent protein kinase, resulting in a decrease in its calcium-mobilizing activity³⁵.

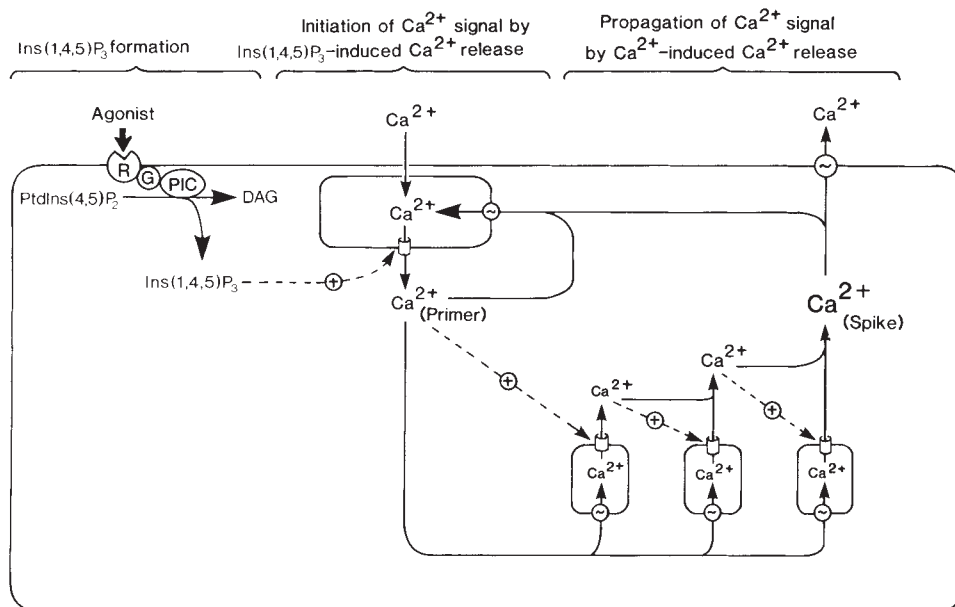
A characteristic feature of the $\text{Ins}(1,4,5)\text{P}_3$ receptor is that it does not desensitize. Any decline in the release of calcium can usually be attributed either to the rapid metabolism of $\text{Ins}(1,4,5)\text{P}_3$ (ref. 23) or to the transfer of the mobilized calcium to an $\text{Ins}(1,4,5)\text{P}_3$ -insensitive pool. A useful analogue for studying calcium mobilization is inositol 1,4,5-trisphosphorothioate which is resistant to metabolism by either the $\text{Ins}(1,4,5)\text{P}_3$ -5-phosphatase (ref. 36) or the $\text{Ins}(1,4,5)\text{P}_3$ -3-kinase (ref. 37) and the lack of desensitization of the $\text{Ins}(1,4,5)\text{P}_3$ receptor is emphasized by the fact that calcium mobilized by this phosphorothioate derivative is not re-sequestered³⁷.

Calcium entry—two inositol phosphates involved?

In addition to mobilizing internal calcium through $\text{Ins}(1,4,5)\text{P}_3$, many agonists can also promote an influx of external calcium. The three main types of calcium channel used to regulate calcium influx are voltage-operated calcium channels, and two agonist-dependent types which are opened either directly through a receptor (receptor-operated channel) or indirectly through some internal diffusible messenger (second messenger-operated channel)³⁸. These calcium channels differ in the way they are activated and, probably, in the very different calcium signals that they generate. Voltage-operated calcium channels, which are usually found in excitable cells, give rapid but brief calcium pulses when activated by membrane depolarization. Opening of receptor-operated channels, however, results in a rapid and maintained elevation in intracellular calcium which is also totally dependent on external calcium. The calcium signal derived from the opening of voltage-operated channels may be amplified by the mobilization of intracellular calcium through a calcium-dependent formation of $\text{Ins}(1,4,5)\text{P}_3$ (ref. 39).

There is considerable uncertainty concerning the mechanism and control of second messenger-operated channels. The amount of calcium flowing through these channels is very small, and they do not display the characteristics of typical channels but seem instead to generate a smooth inward current⁴⁰. When cells are stimulated with agonists operating through these channels, the large initial increase in calcium (due mainly to mobilization of internal calcium) is not maintained and the

FIG. 2 A unified hypothesis of the spatio-temporal aspects of calcium signalling, based on ideas concerning calcium oscillations^{67,79} and the propagation of calcium waves^{57–60}. A typical response begins with agonists generating $\text{Ins}(1,4,5)\text{P}_3$, which then mobilizes calcium from an $\text{Ins}(1,4,5)\text{P}_3$ -sensitive calcium store and also promotes the entry of external calcium (see Fig. 3 for details) to give an initial calcium signal. In some cells, this $\text{Ins}(1,4,5)\text{P}_3$ -induced calcium signal can act as a 'primer' to drive a process of calcium-induced calcium release from the $\text{Ins}(1,4,5)\text{P}_3$ -insensitive pools to produce a spike which might be organized in the form of a wave, so spreading the signal throughout the cell.



concentration usually declines to a low plateau level. Both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ have been implicated in controlling this slow entry of external calcium. Just how these inositol phosphates may function is confused because the route of calcium entry is unknown. The simplest hypothesis is that calcium flows directly into the cytosol through channels in the plasma membrane (Fig. 3a). Such channels could be regulated by $\text{Ins}(1,4,5)\text{P}_3$ as has been reported in lymphocytes⁴¹ and mast cells⁴⁰. An alternative capacitative mechanism is that calcium flows first into the ER before entering the cytosol⁴². This is an autoregulatory mechanism because the calcium content of ER that is closely apposed to the plasma membrane somehow regulates the flow of calcium into the cell (Fig. 3b). Just how calcium traverses both the plasma membrane and the ER membrane is not known but one idea is that it passes through a pore (analogous to a gap junction) linking the two membranes⁴³. Consistent with this hypothesis that the emptying of an internal pool is a prerequisite for entry, is the observation that release of calcium from the internal pool of endothelial cells can precede the entry of external calcium⁴⁴; the control of calcium entry would, therefore, be regulated indirectly by the ability of $\text{Ins}(1,4,5)\text{P}_3$ to discharge the ER of its calcium (Fig. 3b).

This proposed role of the ER may help to explain a possible involvement of $\text{Ins}(1,3,4,5)\text{P}_4$ in regulating calcium entry, because the action of this inositol phosphate seems to require the simultaneous presence of $\text{Ins}(1,4,5)\text{P}_3$ (refs. 11, 45). $\text{Ins}(1,3,4,5)\text{P}_4$, at low concentrations and with a high degree of chemical specificity, increases intracellular calcium concentrations provided that a calcium-mobilizing InsP_3 is present⁴⁵⁻⁴⁷. This is not the result of $\text{Ins}(1,3,4,5)\text{P}_4$ competing for the phosphatase that it shares with $\text{Ins}(1,4,5)\text{P}_3$ which would protect the latter compound⁴⁷. At least some, but not all, of the calcium released comes from outside the cell⁴⁷ and the current best guess is that $\text{Ins}(1,3,4,5)\text{P}_4$ controls the transfer of calcium between intracellular pools¹¹ (Fig. 3c). If some of these pools regulate calcium entry through the capacitative mechanism described above, then this may explain the absolute dependency of calcium entry on $\text{Ins}(1,3,4,5)\text{P}_4$ (ref. 47) (see Fig. 3c). The lack of such a dependency on $\text{Ins}(1,3,4,5)\text{P}_4$ in other experiments⁴⁰ may be explained by the slow reversibility of the effects of $\text{Ins}(1,3,4,5)\text{P}_4$ once they are initiated⁴⁸. Even if $\text{Ins}(1,3,4,5)\text{P}_4$ is essential for inositol phosphate-controlled calcium entry, its contribution to intracellular calcium mobilization is, as yet, unknown. Finally, we should note that the idea of $\text{Ins}(1,3,4,5)\text{P}_4$ linking pools, may also provide an explanation for the increased sequestration of calcium caused by this inositol phosphate in a permeabilized cell preparation⁴⁹.

Clearly, there is still a lot to learn about how second messengers regulate calcium entry to maintain the signalling system during prolonged periods of stimulation. The relatively small influx of calcium through second messenger-operated channels does not normally result in marked elevation of intracellular calcium, but it does drive calcium oscillations in many cells by providing a steady input of calcium which is then

TABLE 1 Calcium wave propagation velocities

Cell type	Temperature (°C)	Propagation velocity ($\mu\text{m s}^{-1}$)	Reference number
Rat cardiac myocytes	23	33	60
Rat cardiac trabeculae	30	74	
	21	50-1,500	58
Hamster egg	30-32	16-28	52
Medaka egg	26	12	66
<i>Xenopus</i> egg	22.5	8-10	57
Sea urchin egg	16.0	5	65
Tracheal epithelium	37	10	85
Endothelial cell	37	50	62
Gonadotropes	22	45-207	*

* D. Leong and M. Thorner, personal communication.

periodically released to give large but transient calcium spikes (see below).

Spatial organization and calcium waves

With the advent of calcium-sensitive indicators and image analysis it has become possible to study how calcium signals are distributed in space and time within a single cell⁵⁰. Calcium signals may either be uniform, or they can initiate in discrete regions from which they may propagate in the form of waves. In bovine adrenal cells, for example, the opening of voltage-operated channels gives a uniform calcium signal, whereas the activation of muscarinic receptors results in a highly localized rise in calcium⁵¹ (Fig. 4). Such discrete initiation sites have been observed in other cells responding to stimuli which hydrolyse inositol lipids, for example, in eggs⁵², adrenal glomerulosa cells⁵³, *Limulus* photoreceptors⁵⁴ and NIH-3T3 cells⁵⁵. This spatial organization of calcium signals may be determined by an unequal distribution either of the receptor mechanisms responsible for generating $\text{Ins}(1,4,5)\text{P}_3$ or of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive calcium pools⁵⁶. Fertilization is an example where the external signal dictates the initiation site of the calcium signal; this begins at the point of sperm-egg fusion from where it spreads as a propagated wave towards the opposite pole^{52,57}. Calcium waves also occur in cardiac muscle⁵⁸, myocytes^{59,60}, gonadotropes⁶¹, endothelial cells⁶² and hepatocytes⁶³. In all these cells, the waves propagate in a non-decremental way at very similar velocities (Table 1), suggesting a common mechanism, which could be a well-ordered system of calcium pools aligned to allow calcium to spread from one pool to another (Fig. 2). In myocytes, the sarcomeric organization of the myofibrils separates the sarcoplasmic reticulum into regular arrays. *Xenopus* eggs also have a regularly spaced arrangement of the ER lined up close to the plasma membrane⁶⁴. The higher density of ER in the animal as compared with the vegetal hemisphere, may explain how the wave propagates faster in the former⁶⁴.

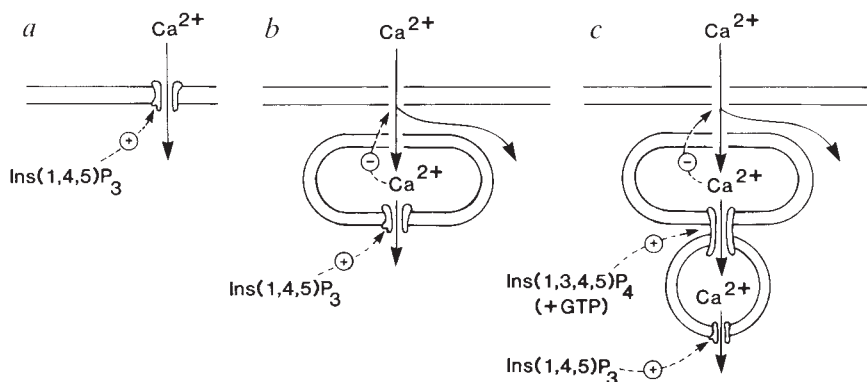


FIG. 3 Summary of the proposed mechanisms by which inositol phosphates control calcium entry into cells. a, $\text{Ins}(1,4,5)\text{P}_3$ controls a calcium channel in the plasma membrane^{40,41}; b, a capacitative model, in which $\text{Ins}(1,4,5)\text{P}_3$ controls a channel in an endomembrane compartment which indirectly regulates calcium entry across the plasma membrane through the operation of a negative-feedback loop⁴²; c, extension of Putney's capacitative model to include a role for $\text{Ins}(1,3,4,5)\text{P}_4$ in controlling the transfer of calcium between pools^{11,45}.

There are two principal models of how calcium waves might spread through cells. One suggestion is that $\text{Ins}(1,4,5)\text{P}_3$ and calcium act as positive regulators of each other⁶⁵. An essential feature of this model is that calcium activates the production of $\text{Ins}(1,4,5)\text{P}_3$. Although an increase in calcium is not obligatory for the activation of phosphoinositidase C, elevating calcium can enhance the formation of $\text{Ins}(1,4,5)\text{P}_3$ in certain cells³⁹. The other model, to us more attractive, is that the wave spreads by a process of calcium-induced calcium release coupled by diffusion as has been suggested for eggs^{52,57,66} and for contractile cells^{58,59}. Calcium elevated at a discrete initiation site diffuses to an adjacent store to stimulate the release of calcium and so on down the array^{58,59} (Fig. 2). Under circumstances where there are multiple initiation sites, separate waves may result, and these can collide and annihilate each other⁵⁹. This indicates that there is a refractory period following the wave⁵⁹, which probably depends on the time required to recharge the calcium stores to the point at which they can once again respond to a calcium signal. We argue below that $\text{Ins}(1,4,5)\text{P}_3$ may play a key role in priming these stores.

Calcium oscillations

The intracellular level of calcium often oscillates, especially when the phosphoinositide pathway is being stimulated^{29,67}. There are many oscillatory patterns, but usually they have the form of a constant baseline which is periodically interrupted by calcium spikes. In contrast to the more classical membrane oscillators which operate through a phasic opening of voltage-operated calcium channels in the plasma membrane, the oscillatory mechanism we are considering here is based on the periodic release of internal calcium (cytosolic oscillators). Most oscillations have a period of between 5 and 60 s (ref. 29) but it is particularly noteworthy that this frequency depends on agonist concentration in some tissues⁶⁸⁻⁷¹ but not others^{72,73}. Whereas the form and frequency of the oscillations vary from cell to cell, they are remarkably constant for individual cells responding to repeated application of a specific agonist, and have, therefore, been referred to as 'fingerprints'⁷¹. Some oscillations cease almost immediately when external calcium is removed⁷⁰ whereas others persist for a considerable time⁶⁷. All these differences suggest that oscillations may be generated by different mechanisms under different circumstances²⁹. In most of the models developed so far, $\text{Ins}(1,4,5)\text{P}_3$ is a key regulator of the periodic release of internal calcium; however, there is considerable controversy over whether the concentration of $\text{Ins}(1,4,5)\text{P}_3$ oscillates⁶⁷.

In receptor-controlled oscillator models, feedback loops that control the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ by phosphoinositidase C are invoked, that cause the concentration of $\text{Ins}(1,4,5)\text{P}_3$ to oscillate. This may be through a negative feedback loop using the DAG/protein kinase C pathway^{29,74}, or calcium may be a positive regulator of $\text{Ins}(1,4,5)\text{P}_3$ formation⁷⁵. In the latter hypothesis, an important consideration is the strong cooperative interaction between $\text{Ins}(1,4,5)\text{P}_3$ and the receptor through which it mobilizes calcium²⁶. A third model^{45,73} invokes the calcium-dependent conversion of $\text{Ins}(1,4,5)\text{P}_3$ to $\text{Ins}(1,3,4,5)\text{P}_4$ (ref. 76), which may act as a feed-forward signal to promote further calcium mobilization (see above). An attractive feature of this model is that the calcium spike will not be maintained because full activation of the $\text{Ins}(1,4,5)\text{P}_3$ -3-kinase would soon exhaust all the $\text{Ins}(1,4,5)\text{P}_3$ and terminate further release because $\text{Ins}(1,3,4,5)\text{P}_4$ cannot mobilize calcium on its own. However, the surge in $\text{Ins}(1,3,4,5)\text{P}_4$ may set the stage for the next transient by helping to load the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pool both by stimulating the re-uptake of calcium into this pool⁴⁹ and by promoting the transfer of calcium between intracellular pools^{11,45}.

Present techniques are not sensitive enough to determine whether $\text{Ins}(1,4,5)\text{P}_3$ is oscillating in single cells. As oscillations can be observed when a variety of cells are injected or perfused with $\text{Ins}(1,4,5)\text{P}_3$, receptor-controlled models seem unlikely, at

least in these systems^{67,77}. In particular, in pancreatic cells, identical oscillations to those produced by acetylcholine were induced by perfusing the cell with the non-metabolizable phosphorothioate derivative of $\text{Ins}(1,4,5)\text{P}_3$, thus making less likely a role for fluctuations in $\text{Ins}(1,4,5)\text{P}_3$ or $\text{Ins}(1,3,4,5)\text{P}_4$ (ref. 77).

Second-messenger controlled models propose that the oscillator resides within the ER/SR system which releases calcium periodically in response to a steady level of $\text{Ins}(1,4,5)\text{P}_3$. One proposal^{33,71,72,78} is that release may become periodic because a negative calcium feedback is known to inhibit both the binding and the calcium-mobilizing action of $\text{Ins}(1,4,5)\text{P}_3$ (refs 15, 33). Another model, based on that discussed above for the initiation and propagation of a calcium wave in *Xenopus* eggs⁵⁷, considers that oscillations arise through an interplay between different calcium pools (Fig. 2). As the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pool empties it primes the $\text{Ins}(1,4,5)\text{P}_3$ -insensitive calcium pool which then begins to release calcium in an oscillatory manner through the

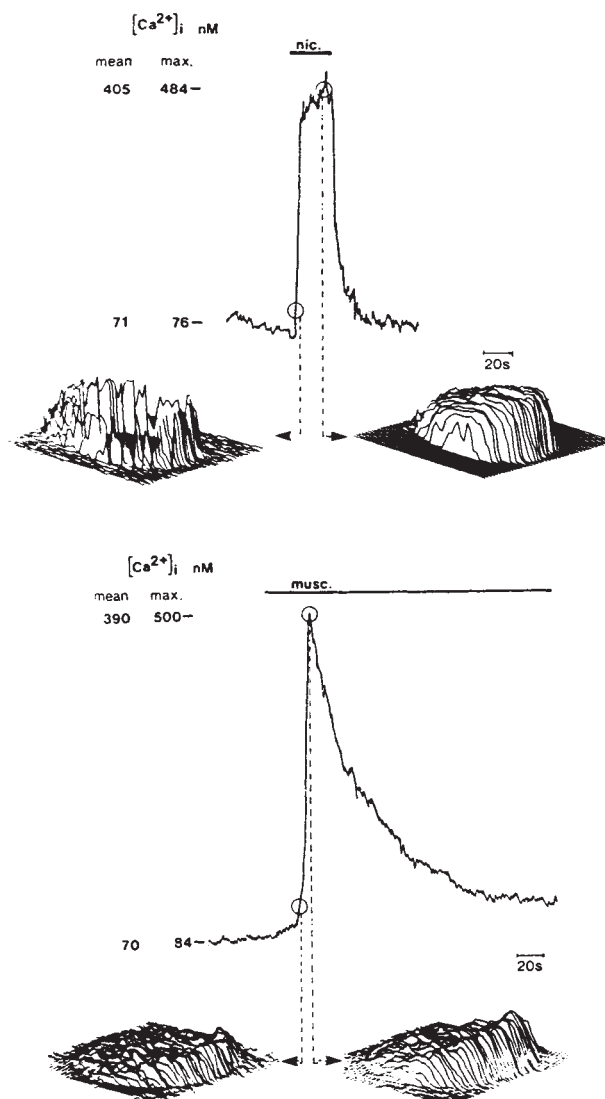


FIG. 4 Spatial organization of calcium signalling in a bovine adrenal chromaffin cell during stimulation with either nicotine (nic) or muscarine (musc). Although these two stimuli gave comparable mean and maximal calcium signals, the contour maps reveal that cellular distributions of calcium during the onset (left) and at the peak of the response (right) are very different. During nicotine stimulation, which results in the opening of voltage-operated channels, calcium rises initially around the periphery and then the interior fills in to give a uniform elevation throughout the cell. On the other hand, the increase in calcium following muscarinic stimulation is highly localized, in the form of a crescent, to one region of the cell. (Unpublished, using data taken from ref. 51).

process of calcium-induced calcium release^{29,67} which has previously been proposed for setting up oscillations in various cell types^{56,59,79-81}.

Just how calcium release is triggered is uncertain. Whatever the activation mechanism is, it must be related in some way to the filling of the internal store to some constant level to account for the observation that both the rate of rise and the amplitude of the individual calcium spikes remain constant despite the large changes in frequency induced by varying agonist concentrations^{69,70,79}. The build-up of calcium in the ER compartment somehow precipitates a sudden release of calcium either because the concentration within the cisternae reaches some critical threshold or because the saturation of the uptake system nullifies its buffering capacity so that cytosolic calcium is elevated, and this provides the activation signal (Fig. 2). The existence of a 'pacemaker' calcium ramp preceding the onset of the calcium spike in endothelial cells supports this latter mechanism⁷⁰.

Models based on calcium-induced release are attractive because this process can account for many of the characteristics of calcium oscillations and also for how these can be induced by stimuli other than those which act through inositol lipid hydrolysis. Because oscillations are apparently a calcium-overload response, repetitive calcium spiking will follow any stimulus which increases intracellular calcium, such as potassium depolarization in sympathetic ganglia⁸⁰ and smooth muscle⁸², or by placing mammalian cardiac muscle in low sodium media or treating it with ouabain⁵⁹. The period between spikes will be determined by how long it takes to charge up (prime) the $\text{Ins}(1,4,5)\text{P}_3$ -insensitive pool. The external concentration of calcium will influence oscillator frequency depending on the extent to which it contributes to the loading of this pool⁶⁷. If the oscillator is reasonably self-contained, that is, most of the calcium in the spike is returned to internal pools, removing external calcium will have little immediate effect. If, on the other hand, most of the calcium is extruded from the cell following each transient, oscillator frequency will be very dependent on external calcium, as it is in myocytes⁵⁹, endothelial cells⁷⁰ and hepatocytes⁷⁹. The remarkable agonist-specific shapes of individual calcium transients in hepatocytes^{74,79} may be explained by agonists having variable effects on the rate of calcium extrusion from cells⁷⁹.

Calcium oscillations can be considered as a transformation of the calcium signal into a digital form whose frequency varies with agonist concentration. Calcium signalling may therefore be frequency-coded and the advantages of using frequency instead of amplitude are discussed in detail elsewhere^{29,68-70}. Such oscillatory activity may not only mediate the action of neurotransmitters and hormones, but may also be important in regulating cell growth. Prolonged oscillations have been described in lymphocytes responding to antigen⁸³ and also during interphase of Swiss-3T3 cells, particularly as the cells exit from mitosis⁸⁴.

A unifying hypothesis

The distribution of calcium signals in space and time seem to be reflections of a common mechanism, and so we can begin to formulate a unified hypothesis^{59,67,79}. Each transient during a calcium oscillation can be spatially organized, as it is often initiated at a discrete site from which it then spreads as a wave throughout the cell. This sequence of events begins with the agonist-stimulated formation of $\text{Ins}(1,4,5)\text{P}_3$ which activates the process of calcium-induced calcium release that is responsible for the spatially-organized calcium transient as discussed above (Fig. 2). Although the significance of the different components may vary from cell to cell, there is every reason to suppose that both oscillations and signal propagation through calcium waves share a common mechanism.

So far we have concentrated on events in a single cell, but many cells are connected together by means of gap junctions

which may allow cells to function in unison with regard to both oscillatory activity and wave propagation. Evidence that a calcium wave may travel from one cell to the next has come from studies of the ciliated tracheal epithelium⁸⁵. Mechanical excitation of one cell accelerates its ciliary beat through an increase in intracellular calcium. A wave of excitation then spreads to neighbouring cells at a rate of $\sim 10\text{--}15\ \mu\text{m s}^{-1}$ (ref. 85). This rate is similar to that for the intracellular waves described earlier (Table 1), so it seems that propagation may spread from one cell to the next, provided that sufficient components of the wave ($\text{Ins}(1,4,5)\text{P}_3$ or calcium) can flow through the gap junctions. Gap junctions are known to provide an avenue for intercellular stimulation by cyclic AMP (ref. 86) and there is evidence that both $\text{Ins}(1,4,5)\text{P}_3$ and calcium can flow down a chain of hepatocytes, sometimes in the form of a wave⁶³. Intercellular communication of $\text{Ins}(1,4,5)\text{P}_3$ or calcium may also explain the synchronized calcium oscillations described in endothelial monolayers⁸⁷ and the oscillatory release of calcium from the intact liver following hormonal stimulation⁸⁸. It is unclear whether such synchronization arises because all the cells are oscillating in phase, or whether there is a 'pacemaker' centre which entrains neighbouring oscillators by passing information along gap junctions, but either way, the spatiotemporal hypothesis of calcium signalling may help to explain the way in which cell populations communicate with each other to produce synchronous responses.

Why so many inositol phosphates?

Cells contain a bewildering array of inositol phosphates¹⁰⁻¹² which seem to fall into two functional groups (Fig. 1): those whose levels change in response to agonist stimulation and thus have functions related to intracellular signalling, and the agonist-insensitive group concerned with the synthesis of InsP_5 and InsP_6 whose levels, if they change, do so comparatively slowly during stimulation. In the agonist-sensitive group, the first complication is that, in addition to $\text{Ins}(1,4,5)\text{P}_3$ being formed by phosphoinositidase C activity on $\text{PtdIns}(4,5)\text{P}_2$, some cyclic inositol trisphosphate ($\text{Ins}(1:2\text{cyc},4,5)\text{P}_3$) is also released⁸⁹. $\text{Ins}(1:2\text{cyc},4,5)\text{P}_3$ is formed very slowly^{90,91}, but as it is a poor substrate for the phosphatase and kinase that metabolize $\text{Ins}(1,4,5)\text{P}_3$ (refs 11, 12), it can eventually accumulate to reach a concentration similar to that of $\text{Ins}(1,4,5)\text{P}_3$, after prolonged stimulation⁹⁰. Although $\text{Ins}(1:2\text{cyc},4,5)\text{P}_3$ can mobilize calcium⁸⁹, its potency is probably at least an order of magnitude less than $\text{Ins}(1,4,5)\text{P}_3$ (ref. 92). The available evidence, therefore, indicates that cells cannot avoid making cyclic inositol phosphates (because of the way phosphoinositidase C works) but that they are unlikely to use them for any physiological purpose.

Much of the remaining proliferation of agonist-sensitive inositol phosphates originates from the metabolism of $\text{Ins}(1,4,5)\text{P}_3$ being through two separate pathways (Fig. 1). It can be sequentially dephosphorylated to free inositol (enzymes 10 and 11, Fig. 1) or it can be phosphorylated to $\text{Ins}(1,3,4,5)\text{P}_4$ by an $\text{Ins}(1,4,5)\text{P}_3$ -3-kinase (enzyme 15, Fig. 1). The latter is strongly activated in *v-src* transformed cells resulting in a seven-fold elevation in the level of an InsP_4 (ref. 93). The hydrolysis of $\text{Ins}(1,3,4,5)\text{P}_4$ to $\text{Ins}(1,3,4)\text{P}_3$ is a straightforward reaction, but much of the complexity arises because the latter can be hydrolysed back to inositol by separate routes (Fig. 1). What is the significance of this complexity? At this stage we can offer two extreme viewpoints. The complexity could arise because one or more of the breakdown products themselves have physiological functions. One could argue teleologically that this would be undesirable, because the only route of synthesis of any of these other inositol phosphates is through a known second messenger, $\text{Ins}(1,4,5)\text{P}_3$, so an increase in another putative second messenger would always have to be associated with a rise in calcium. We think that it is more likely that the complexities are simply an inevitable result of the 3-phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ to form $\text{Ins}(1,3,4,5)\text{P}_4$: if the cells are coping

with the formation of two second messengers—Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄—rather than one, more enzymes will be required to catabolize these, and so a complex pattern of intermediates cannot be avoided¹¹.

InsP₅ and InsP₆

The metabolism of InsP₅ and InsP₆ occurs through pathways which are largely separate from the agonist-sensitive pathways (Fig. 1). The main route by which these compounds are made in animal cells is not yet clear and may involve separate pathways for InsP₅ and InsP₆. In the case of InsP₅, a pathway exists from agonist-sensitive inositol phosphates through the phosphorylation of Ins(1,3,4)P₃ to Ins(1,3,4,6)P₄ and thence to Ins(1,3,4,5,6)P₅ (ref. 96) (Fig. 1). However, the main route of synthesis of this inositol phosphate is probably through Ins(3,4,5,6)P₄, which has long been known to be a constituent in avian erythrocytes along with InsP₅ (ref. 95) and which also exists in mammalian cells, together with an active and specific kinase which converts it to InsP₅ (ref. 94). WRK-1 cells have very high levels of Ins(3,4,5,6)P₄ which increase slowly following stimulation with vasopressin⁹⁷. But the origin of Ins(3,4,5,6)P₄ is, as yet, unknown; that is, we do not know if it is formed from phosphorylation of inositol or indirectly from Ins(1,4,5)P₃. InsP₆ is very likely to be generated by phosphorylation of inositol. InsP₅ and InsP₆ incorporate radiolabel more slowly than 'receptor-generated' inositol phosphates, but they can eventually incorporate [³H]inositol up to a very high level⁹⁸, consistent with their high (millimolar) mass levels in the cell⁹⁹ (compare for example the micromolar levels of Ins(1,4,5)P₃ (ref. 100)). The proportion of InsP₅ to InsP₆ changes markedly when HL60 cells begin to differentiate¹⁰¹. The overall picture we have of these compounds is, therefore, one of a high resting level which, at least for Ins(1,3,4,5,6)P₅, can perhaps be changed after prolonged stimulation by synthesis from Ins(1,3,4)P₃.

InsP₅ and InsP₆ are therefore generally assumed to have a 'housekeeping' function rather than being acute second messengers. Speculations about their function have abounded, but perhaps the most promising for InsP₆ centres around its unique anti-oxidant properties¹⁰². Recently it has been suggested that InsP₅ and InsP₆ are extracellular signals, neurotransmitters¹⁰³ for example, but even if correct, this exciting discovery does not help to clarify their intracellular roles.

Lipid diversity and enzyme heterogeneity

Phosphatidylinositol has a rich diversity of forms and functions within the cell, as do the enzymes associated with its metabolism. Apart from its structural role as part of the lipid bilayer, PtdIns is a precursor of second messengers and is a membrane anchor for a great variety of cell surface proteins¹⁰⁴. This functional diversity is associated with structural variations of both the inositol head group and the fatty-acid tail regions. For example, the PtdIns used for protein anchoring has a fatty-acid composition rich in myristic acid, whereas that attached to PtdIns(4,5)P₂ is predominantly stearoyl-arachidonyl in composition.

A major function of inositol lipids is the one principally under discussion here, to supply second messengers such as Ins(1,4,5)P₃ and DAG in response to calcium-mobilizing agonists¹⁻⁷. For this function, PtdIns is phosphorylated to PtdIns(4)P and then to PtdIns(4,5)P₂; the latter is the precursor used for signal transduction (Figs 1 and 2). The recent discovery of a new lipid, PtdIns(3)P, has emerged from work being carried out on the function of the proto-oncogene *c-src* (ref. 105). The product of *c-src* in association with the middle T antigen of SV40, acts to phosphorylate (and thus possibly to activate) type I PtdIns kinase, which is distinct from the type II enzyme responsible for forming PtdIns(4)P, not least because it phosphorylates PtdIns on the 3-position to give PtdIns(3)P (Fig. 1). A possible role for type I kinase in cell proliferation¹⁰⁵ has recently received strong support from studies which show that platelet-derived growth factor (PDGF) receptors that are

deficient in their ability to associate with this kinase fail to stimulate growth¹⁰⁶. There is also a report of the transient appearance of a PtdIns containing 4 phosphates (possibly PtdIns(3,4,5)P₃) in stimulated neutrophils¹⁰⁷ and of a possible PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ in PDGF-stimulated fibroblasts¹⁰⁸. It is tempting to speculate whether such novel lipids fulfil some of the other proposed functions of PtdIns(4,5)P₂, such as remodelling the cytoskeleton^{109,110}, without compromising the established function of PtdIns(4,5)P₂ as a precursor for DAG and the inositol polyphosphates.

The protein-anchoring function of PtdIns glycans serves as a mechanism for quickly converting a protein which does not have a predominantly hydrophobic region, into a membrane-bound form. It also provides a means for the rapid release of proteins from membranes by reactions catalysed by phospholipase C, as is observed during the change of the protein coat in trypanosomes¹⁰⁴. Also, the insulin-sensitive hydrolysis of a glycosylated PtdIns releases an inositol phosphate glycan which might be a second messenger for some of the actions of insulin¹⁰⁴.

The growing diversity of the inositol lipids is more than matched by the number of enzymes now known to be responsible for both the initial production and subsequent metabolism of Ins(1,4,5)P₃ (Fig. 1). This complexity is increased because there are different molecular forms of many of these enzymes, for example Ins(1,4,5)P₃-5-phosphatase¹¹¹ (enzymes 10 and 11, Fig. 1) and PtdIns synthetase¹¹² (enzyme 1, Fig. 1). The recent discovery of a possible function of inositol lipids in the nucleus¹¹³ may itself spawn an entirely new family of enzymes. Indeed, Sylvia *et al.*¹¹⁴ have reported that Ins(1,4)P₂ or its parent lipid PtdIns(4)P can specifically activate a DNA polymerase.

The molecular basis for this enzyme heterogeneity is being studied by genetic techniques which have already revealed that protein kinase C is a heterogeneous enzyme family, with a distinct, and in some cases very precise, tissue specificity². Phosphoinositidase C also exists in a remarkable number of different forms¹¹⁵ and at least one of these may have coding regions resembling the tyrosine kinase-related oncogenes. Studies on the *Drosophila NorpA* mutant, which is blind but otherwise normal, illustrate the degree to which this phosphoinositidase C family may be tissue-specific. When Yoshioka and colleagues showed that these flies lack phosphoinositidase C in their eyes and suggested that *NorpA* might be a structural gene for this enzyme¹¹⁶, the idea was received with some scepticism because it would require a phosphoinositidase C gene specific only for photoreceptors. The recent genetic confirmation that *NorpA* does code for a protein with very close similarities to known phosphoinositidase C enzymes, and the observation that transcripts of the gene were localized to the eye and a small region of the brain, suggests that such tissue specificity might indeed exist¹¹⁷.

Lithium

When administered to intact organisms, lithium induces subtle alterations in neural activity (for example, in manic-depressive illness and diurnal rhythms) and early development (teratogenesis). Lithium is known to reduce the supply of inositol, the key substrate for the phosphoinositide cascade, by inhibiting some of the enzymes (enzymes 13 and 14, Fig. 1), which hydrolyse the inositol phosphates. The 'inositol depletion hypothesis' may explain the neural and developmental effects of lithium on the basis that it inhibits signal transduction indirectly by slowing down the supply of the precursor lipid required to generate messengers such as Ins(1,4,5)P₃ and DAG (ref. 118). The subtlety of the action of lithium is that it seems to be selective; it ignores those receptors which are operating normally, but 'searches out' and dampens down those which are overactive. This selectivity is enhanced by the inhibitory action of lithium being uncompetitive¹¹⁹ with the result that its effect is progressively enhanced as the level of substrate increases. It is this selective action against overactive receptors

which is the key feature of the inositol depletion hypothesis. Those hypotheses which attempt to explain how lithium might act through a direct effect on signal-transducing elements (adenylyl cyclase or G proteins) cannot satisfactorily explain why lithium has no acute effect on a variety of cellular responses, nor why it has little or no effect on people not suffering from manic-depressive illness. However, it is not presently clear how the apparent tissue (brain)- and agonist (carbachol)-specific inhibition by lithium of inositol phosphate formation¹²⁰, is related to its effects on inositol phosphate phosphatases, nor to the influence of lithium on manic depression.

The teratogenic effect of lithium has been investigated in *Xenopus* where its effect is to respecify the dorsal-ventral axis which is fixed at the time of fertilization. This polarity in the early embryo is based on some positional variable or 'organizer' set up in the form of a gradient along the dorsal-ventral axis. More specifically, Busa¹²¹ has proposed that phosphoinositide-derived messengers such as $\text{Ins}(1,4,5)\text{P}_3$ or DAG might be the morphogens responsible for early pattern formation. On the basis of the inositol depletion hypothesis, the action of lithium can be interpreted by supposing that it flattens out a normal gradient of phosphoinositide turnover which is postulated to be high in the ventral pole and low in the dorsal pole. Conversely, UV-irradiation which promotes ventralization may produce a uniformly high level of phosphoinositide turnover.

A gradient of phosphoinositide turnover along the dorsal-ventral axis is entirely consistent with the distribution of junctional permeability in the early embryo. Activation of the phosphoinositide messenger pathway, particularly the DAG protein kinase C branch, is known to decrease the permeability of gap junctions¹²². It is significant, therefore, that transfer of the dye Lucifer Yellow between cells in the ventral region is less than in the dorsal region¹²³. If pattern formation depends upon messengers such as $\text{Ins}(1,4,5)\text{P}_3$ and calcium, which can diffuse between cells (see above), then this reduction in junctional permeability in the ventral pole may serve to confine these morphogens to this region. Treatment with lithium gives a uniformly high level of dye transfer, whereas UV-irradiation reduces transfer¹²³ and this is exactly in line with the proposal that these two treatments even out the gradient of phosphoinositide turnover to levels which are either uniformly low (lithium) or high (UV-irradiation).

The hypothesis based on a gradient of phosphoinositide turnover is also consistent with experiments where lithium has been injected into one or other pole^{121,124,125}. Dorsalization induced by injecting lithium specifically into the ventral pole can be prevented if the lithium is co-injected with *myo*-inositol (but not *epi*-inositol)¹²⁵, an observation that provides strong support for the inositol depletion hypothesis. Furthermore, it suggests that a gradient of phosphoinositide messengers may be pivotal in pattern formation during embryogenesis.

Conclusion

In reviewing our current understanding of the role of $\text{Ins}(1,4,5)\text{P}_3$ in regulating physiological functions of cells *in vivo*, we have highlighted many complexities: multiple calcium pools, the interrelationship between calcium mobilization and entry, and several possible mechanisms of generating calcium oscillations and waves. Pervading all these aspects to an, as yet unknown degree is the probable helper function of $\text{Ins}(1,3,4,5)\text{P}_4$. These subtleties and complexities suggest a reason why the inositol phosphate signalling system is so widespread in biology. It gives to cells the ability to do so much more than just to raise or lower calcium levels. This in turn provides the means to generate an enormous variety of cellular responses from one essentially straightforward set of signals. For example, a cell may distinguish one receptor from another, even though both are working through inositol phosphates. It may use the same signals to control short-term functions (such as secretion or metabolism) and long-term functions (such as

growth), and the interactions between different intracellular signals could be virtually limitless in their variety.

The complicated biochemistry of inositol phosphates illustrates the extraordinary diversity of compounds into which *myo*-inositol can be incorporated. Here we have limited our survey mostly to vertebrates, but many more inositol-containing compounds exist in other organisms, particularly in plants. We know very little about how these many compounds are formed, and we are just beginning to fathom functions for only a few of them. In vertebrates, a main challenge for the future is to reveal the wider implications of the phosphoinositide signalling system in the control of complex biological processes, such as early development and the neural mechanisms which underly behaviour. □

Michael J. Berridge is at the AFRC Unit of Insect Neurophysiology and Pharmacology, Department of Zoology, Downing Street, Cambridge CB2 3EJ, UK. Robin F. Irvine is at the AFRC Institute of Animal Physiology and Genetics Research, Cambridge Research Station, Babraham Hall, Cambridge CB2 4AT, UK

- Berridge, M. J. & Irvine, R. F. *Nature* **312**, 315-321 (1984).
- Nishizuka, Y. *Nature* **334**, 661-665 (1988).
- Berridge, M. J. *A. Rev. Biochem.* **56**, 159-193 (1987).
- Hokin, L. E. *A. Rev. Biochem.* **54**, 205-235 (1985).
- Abdel-Latif, A. *A. Pharmac. Rev.* **38**, 227-272 (1986).
- Michell, R. H., Drummond, A. H. & Downes, C. P. (eds) *Inositol Lipids in Cell Signalling* (Academic, London, 1989).
- Berridge, M. J. & Michell, R. H. *Phil. Trans. R. Soc. B.* **320**, 235-436 (1988).
- Parthasarathy, R. & Eisenberg, F. *Biochem. J.* **235**, 313-322 (1986).
- Agranoff, B. W. *Life Sci.* **32**, 2047-2054 (1983).
- Shears, S. B. *Biochem. J.* **260**, 313-324 (1989).
- Irvine, R. F., Moor, R. M., Pollock, W. K., Smith, P. M. & Wreggett, K. A. *Phil. Trans. R. Soc. B* **320**, 281-298 (1988).
- Majerus, P. W. *et al. J. Biol. Chem.* **263**, 3051-3054 (1988).
- Streb, H., Bayerdorffer, E., Hasse, W., Irvine, R. F. & Schulz, I. *J. Membrane Biol.* **81**, 241-253 (1984).
- Thévenod, F. *et al. J. Membrane Biol.* **109**, 173-186 (1989).
- Suppattapone, S., Worley, P. F., Baraban, J. M. & Snyder, S. H. *J. Biol. Chem.* **263**, 1530-1534 (1988).
- Ross, C. A. *et al. Nature* **339**, 468-470 (1989).
- Volpe, P. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 1091-1095 (1987).
- Chueh, S.-H. & Gill, D. L. *J. Biol. Chem.* **261**, 13883-13886 (1986).
- Bourne, H. *Cell* **53**, 669-671 (1988).
- Ghosh, T. K., Mullaney, J. M., Tarazi, F. I. & Gill, D. L. *Nature* **340**, 236-239 (1989).
- Strupish, J., Cooke, A. M., Potter, B. V. L., Gigg, R. & Nahorski, S. R. *Biochem. J.* **253**, 901-905 (1988).
- Taylor, C. W., Berridge, M. J., Brown, K., Cooke, A. M. & Potter, B. V. L. *Biochem. biophys. Res. Commun.* **150**, 626-632 (1988).
- Stauderman, K. A., Harris, G. D. & Lovenberg, W. *Biochem. J.* **255**, 677-683 (1988).
- Hill, T. D., Berggren, P.-O. & Boynton, A. L. *Biochem. biophys. Res. Commun.* **149**, 897-901 (1987).
- Ehrlich, B. E. & Watras, J. *Nature* **336**, 583-586 (1988).
- Meyer, T., Holowska, D. & Stryer, L. *Science* **240**, 653-656 (1988).
- Walker, N. L., Somlyo, A. V., Goldman, Y. E., Somlyo, A. P. & Trentham, D. R. *Nature* **327**, 249-251 (1987).
- Parker, I. J. *J. Physiol., Lond.* **407**, 95P (1988).
- Berridge, M. J., Cobbold, P. H. & Cuthbertson, K. S. R. *Phil. Trans. R. Soc. B.* **320**, 325-343 (1988).
- Seiler, S. M., Arnold, A. J. & Stanton, H. C. *Biochem. Pharmac.* **36**, 3331-3337 (1987).
- Shah, J. & Pant, H. C. *Biochem. J.* **250**, 617-620 (1988).
- Joseph, S. K. & Williamson, J. R. *J. Biol. Chem.* **261**, 14658-14664 (1986).
- Joseph, S. K., Rice, H. L. & Williamson, J. R. *Biochem. J.* **258**, 261-265 (1989).
- Danoff, S. K., Suppattapone, S. & Snyder, S. H. *Biochem. J.* **254**, 701-705 (1988).
- Suppattapone, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 8747-8750 (1988).
- Willcocks, A. L., Potter, B. V. L., Cooke, A. M. & Nahorski, S. R. *Eur. J. Pharm.* **155**, 181-183 (1988).
- Taylor, C. W., Berridge, M. J., Cooke, A. M. & Potter, B. V. L. *Biochem. J.* **259**, 645-650 (1989).
- Pozzan, T. & Meldolesi, J. *Exp. Cell Res.* **171**, 271-283 (1987).
- Eberhard, D. A. & Holz, R. W. *Trends Neurosci.* **11**, 517-520 (1988).
- Penner, R., Matthews, G. & Neher, E. *Nature* **334**, 499-504 (1988).
- Kuno, N. & Gardner, P. *Nature* **326**, 301-304 (1987).
- Putney, J. W. *Cell Calcium* **7**, 1-12 (1986).
- Merritt, J. E. & Rink, T. J. *J. Biol. Chem.* **262**, 4958-4960 (1987).
- Hallam, T. J., Jacob, R. & Merritt, J. E. *Biochem. J.* **255**, 179-184 (1988).
- Irvine, R. F. in *Inositol Lipids in Cell Signalling* (eds Michell, R. H., Drummond, A. H. & Downes, C. P.) 135-161 (Academic, London, 1989).
- Irvine, R. F. & Moor, R. M. *Biochem. J.* **240**, 917-920 (1986).
- Changya, L., Gallacher, D. V., Irvine, R. F., Potter, B. V. L. & Petersen, O. H. *J. Membrane Biol.* **109**, 85-93 (1989).
- Changya, L., Gallacher, D. V., Irvine, R. F. & Petersen, O. H. *FEBS Lett.* **251**, 43-48 (1989).
- Hill, T. D., Dean, N. M. & Boynton, A. L. *Science* **242**, 1176-1178 (1988).
- Tsien, R. Y. & Poenie, M. *Trends biochem. Sci.* **11**, 450-455 (1986).
- O'Sullivan, A. J., Cheek, T. R., Moreton, R. B., Berridge, M. J. & Burgoyne, R. D. *EMBO J.* **8**, 401-411 (1989).
- Miyazaki, S. *et al. Dev. Biol.* **118**, 259-267 (1986).
- Connor, J. A., Cornwall, M. C. & Williams, G. H. *J. Biol. Chem.* **262**, 2919-2927 (1987).
- Payne, R. & Fein, A. J. *J. Cell Biol.* **104**, 933-937 (1987).
- Benjamin, C. W., Connor, J. A., Tarpley, W. G. & Gorman, R. R. *Proc. natn. Acad. Sci. U.S.A.* **85**, 4345-4349 (1988).
- Berridge, M. J. *J. Physiol., Lond.* **403**, 589-599 (1988).
- Busa, W. B., Ferguson, J. E., Joseph, S. K., Williamson, J. R. & Nuccitelli, R. *J. Cell Biol.* **101**, 677-682 (1985).
- Mulder, B. J. M., de Tombe, P. P. & ter Keurs, H. E. D. J. *J. gen. Physiol.* **93**, 943-961 (1989).

59. Lakatta, E. G., Capogrossi, M. C., Spurgeon, H. A. & Stern, M. D. in *Cell Calcium Metabolism* (ed. Fiskum, G.) 529-543 (Plenum, New York, 1989).
60. Kort, A. A., Capogrossi, M. C. & Lakatta, E. G. *Circulation Res.* **57**, 844-855 (1985).
61. Leong, D. A. *et al. J. Cell Biol.* **107**, 498a (1988).
62. Jacob, R. *Cell Calcium* (in the press).
63. Saez, J. C., Connor, J. A., Spray, D. C. & Bennett, M. V. L. *Proc. natn. Acad. Sci. U.S.A.* **86**, 2708-2712 (1989).
64. Gardiner, D. M. & Grey, R. D. *J. Cell Biol.* **96**, 1159-1163 (1983).
65. Swann, K. & Whitaker, M. *J. Cell Biol.* **103**, 2333-2342 (1986).
66. Gilkey, J. C., Jaffe, L. F., Ridgeway, E. B. & Reynolds, G. T. *J. Cell Biol.* **76**, 448-466 (1978).
67. Berridge, M. J. & Galione, A. *FASEB J.* **2**, 3074-3082 (1988).
68. Rapp, P. E. & Berridge, M. J. *J. exp. Biol.* **93**, 119-132 (1981).
69. Woods, N. M., Cuthbertson, K. S. R. & Cobbold, P. H. *Nature* **319**, 600-602 (1986).
70. Jacob, R., Merritt, J. E., Hallam, T. J. & Rink, T. J. *Nature* **335**, 40-45 (1988).
71. Prentki, M. *et al. J. biol. Chem.* **263**, 11044-11047 (1988).
72. Gray, P. T. A. *J. Physiol., Lond.* **406**, 35-53 (1989).
73. Yule, D. I. & Gallacher, D. V. *FEBS Lett.* **239**, 358-362 (1988).
74. Woods, N. M., Cuthbertson, K. S. R. & Cobbold, P. H. *Cell Calcium* **8**, 79-100 (1987).
75. Meyer, T. & Stryer, L. *Proc. natn. Acad. Sci. U.S.A.* **85**, 5051-5055 (1988).
76. Biden, T. J. & Wollheim, C. B. *J. biol. Chem.* **261**, 11931-11934 (1986).
77. Wakui, M., Potter, B. V. L. & Petersen, O. H. *Nature* **339**, 317-320 (1989).
78. Payne, R., Walz, B., Levy, S. & Fein, A. *Phil. Trans. R. Soc. B.* **320**, 359-379 (1988).
79. Rooney, T. A., Sass, E. J. & Thomas, A. P. *J. biol. Chem.* **264** (in the press).
80. Kuba, K. *J. Physiol., Lond.* **298**, 251-269 (1980).
81. Miyazaki, S. *J. Cell Biol.* **106**, 345-353 (1988).
82. Ohya, Y. *et al. Pflügers Arch.* **412**, 382-389 (1988).
83. Wilson, H. A., Greenblatt, D., Poenie, M., Finkelman, F. D. & Tsien, R. Y. *J. exp. Med.* **166**, 601-606 (1987).
84. Tombes, R. M. & Boris, G. G. *J. Cell Biol.* **109**, 627-636 (1989).
85. Sanderson, M. J., Chow, I. & Dirksen, E. R. *Am. J. Physiol.* **254**, C63-C74 (1988).
86. Lawrence, T. S., Beers, W. H. & Gilula, N. B. *Nature* **272**, 501-506 (1978).
87. Sage, S. O., Adams, D. J. & van Breemen, C. *J. biol. Chem.* **264**, 6-9 (1989).
88. Graf, E., Empson, K. L. & Eaton, J. W. *J. biol. Chem.* **262**, 11647-11650 (1987).
89. Wilson, D. B. *et al. J. biol. Chem.* **260**, 13496-13501 (1985).
90. Sekar, M. C., Dixon, J. E. & Hokin, L. E. *J. biol. Chem.* **262**, 340-344 (1987).
91. Wong, N. S., Barker, C. J., Shears, S. B., Kirk, C. J. & Michell, R. H. *Biochem. J.* **252**, 1-5 (1988).
92. Willcocks, A. L., Strupish, J., Irvine, R. F. & Nahorski, S. R. *Biochem. J.* **257**, 297-300 (1989).
93. Johnson, R. M., Wasilenko, W. J., Mattingly, R. R., Webor, M. J. & Garrison, J. C. *Science* (in the press).
94. Stephens, L. R. *et al. Biochem. J.* **249**, 283-292 (1988).
95. Johnson, L. F. & Tate, M. E. *Canadian J. Chem.* **47**, 63-73 (1969).
96. Stephens, L. R., Hawkins, P. T., Barker, C. J. & Downes, C. P. *Biochem. J.* **253**, 721-733 (1988).
97. Barker, C. J., Morris, A. J., Kirk, C. J. & Michell, R. H. *Biochem. Soc. Trans.* **16**, 984-985 (1988).
98. Tilly, B. C. *et al. Biochem. J.* **244**, 129-135 (1987).
99. Martin, J.-B., Foray, M.-F., Klein, G. & Satre, M. *Biochim. biophys. Acta* **931**, 16-25 (1987).
100. Bradford, P. G. & Rubin, R. P. *J. biol. Chem.* **261**, 15644-15647 (1986).
101. French, P. J., Bunce, C. M., Brown, G., Creba, J. A. & Michell, R. H. *Biochem. Soc. Trans.* **16**, 985-986 (1988).
102. Graf, E., Mahoney, J. R., Bryant, R. G. & Eaton, J. W. *J. biol. Chem.* **259**, 3620-3624 (1987).
103. Vallejo, M., Jackson, T., Lightman, S. & Hanley, M. R. *Nature* **330**, 656-658 (1987).
104. Low, M. A. & Saltiel, A. R. *Science* **239**, 268-275 (1988).
105. Whitman, M. & Cantley, L. *Biochim. biophys. Acta* **948**, 327-344 (1989).
106. Coughlin, S. R., Escobedo, J. A. & Williams, L. J. *Science* **243**, 1192-1194 (1989).
107. Traynor-Kaplan, A. E., Harris, A. L., Thompson, B. L., Taylor, P. & Sklar, L. A. *Nature* **334**, 353-356 (1988).
108. Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P. & Cantley, L. C. *Cell* **57**, 167-175 (1989).
109. Lassing, I. & Lindberg, U. *Expl. Cell Res.* **174**, 1-15 (1988).
110. Janney, P. A., Iida, K., Yin, H. L. & Stossel, R. P. *J. biol. Chem.* **262**, 12228-12236 (1987).
111. Hansen, C. A., Johanson, R. A., Williamson, M. T. & Williamson, J. R. *J. biol. Chem.* **262**, 17319-17326 (1988).
112. Cubbitt, A. B. & Gershengorn, M. C. *Biochem. J.* **257**, 639-644 (1989).
113. Cocco, L. *et al. Biochem. biophys. Res. Commun.* **154**, 1266-1272 (1988).
114. Sylvia, V., Curtin, G., Norman, J., Stec, J. & Busbee, D. *Cell* **54**, 651-658 (1988).
115. Rhee, S. G., Suh, S.-G., Ryu, S.-H. & Lee, S. Y. *Science* **244**, 546-550 (1989).
116. Yoshioka, T., Inoue, H. & Hotta, Y. *J. Biochem., Tokyo* **97**, 1251-1254 (1985).
117. Bloomquist, B. T. *et al. Cell* **54**, 723-733 (1988).
118. Berridge, M. J., Downes, C. P. & Hanley, M. R. *Biochem. J.* **206**, 587-595 (1982).
119. Hallcher, L. M. & Sherman, W. R. *J. biol. Chem.* **255**, 10896-10901 (1980).
120. Batty, I. R. & Nahorski, S. R. *Biochem. J.* **247**, 797-800 (1987).
121. Busa, W. B. *Phil. Trans. R. Soc. B.* **320**, 415-426 (1988).
122. Randriamampita, C., Giaume, C., Neyton, J. & Trautmann, A. *Pflügers Arch.* **412**, 462-468 (1988).
123. Nagajski, D. J., Guthrie, S. C., Ford, C. C. & Warner, A. E. *Development* **105**, 747-752 (1989).
124. Kao, K. R., Masin, Y. & Elinson, R. P. *Nature* **332**, 371-373 (1986).
125. Busa, W. B. & Gimlich, R. D. *Dev. Biol.* **132**, 315-324 (1989).

A 42K outer-membrane protein is a component of the yeast mitochondrial protein import site

Dietmar Vestweber, Josef Brunner*, Alison Baker† & Gottfried Schatz

Biocenter, University of Basel, CH-4056 Basel, Switzerland

* Swiss Federal Institute of Technology (ETH), CH-8092 Zürich, Switzerland

An engineered precursor protein that sticks in the import site of isolated yeast mitochondria can be specifically photo-crosslinked to a mitochondrial outer-membrane protein of relative molecular mass 42,000 (42K). This protein (termed import-site protein 42 or ISP 42) is exposed on the mitochondrial surface; antibodies against it block protein import into mitochondria. ISP 42 is the first identified component of the putative transmembrane machinery that imports proteins into mitochondria.

MITOCHONDRIA take up almost all of their proteins from the cytosol¹. One of the earliest steps in this process is the interaction of protein precursors with receptor-like proteins on the mitochondrial surface^{2,3}; none of these proteins has so far been unambiguously identified, although it has been demonstrated

that protein import into isolated yeast mitochondria is inhibited by antibodies or Fab fragments raised against a group of outer membrane proteins⁴ of relative molecular mass (M_r) 45K. It has also been reported that a 30K protein of mammalian mitochondria⁵ or of chloroplasts⁶ interacts with a chemically synthesized targeting peptide specific for the corresponding organelle. There is no direct evidence, however, that any of these polypeptides are components of the protein import machinery.

We report here that a 42K protein (ISP 42) of the yeast mitochondrial outer membrane can be specifically crosslinked to a precursor protein which is stuck in the import sites and that antibodies against ISP inhibit import of precursors into mitochondria. ISP 42 is thus a component of the mitochondrial protein-import system.

The translocation intermediate

The chimaeric precursor⁷, used to investigate the import site, consisted of a fusion protein⁸ with the C terminus crosslinked to bovine pancreatic trypsin inhibitor (BPTI). The fusion protein contained the presequence of yeast cytochrome oxidase subunit IV (coxIV a mitochondrial inner-membrane protein) —joined to the N terminus of mouse dihydrofolate reductase (DHFR a

† Present address: University of Cambridge, Department of Biochemistry, Cambridge CB2 1QW, UK

cytosolic protein). The DHFR moiety had been modified⁷ so that its single cysteine was at the C terminus. This modified coxIV-DHFR fusion protein can be over-expressed in *Escherichia coli*, purified, and covalently linked through its C-terminal cysteine to BPTI using a bifunctional crosslinker. If the resulting chimaeric protein is added to isolated mitochondria, it becomes stuck across both mitochondrial membranes; the DHFR moiety is in the matrix, the presequence is cleaved off by the matrix-localized protease and the BPTI moiety remains outside the mitochondria⁷. Mitochondria that have accumulated more than 40 pmol of this transmembranous intermediate per mg protein, no longer import authentic precursor proteins, indicating that the partly translocated chimaeric precursor remains stuck in the mitochondrial import sites. On subfractionation of the mitochondria, the partly translocated precursor is recovered with a sub-mitochondrial fraction enriched in contact sites between the two mitochondrial membranes⁹. The stuck chimaeric protein is thus a convenient and stable tag for mitochondrial protein-import sites.

To crosslink the stuck precursor to components in its vicinity, we placed a photoreactive group between its two major domains. In the stuck precursor, this group should be ideally situated within the membrane junction. To this end, the trifunctional crosslinker shown in Fig. 1 was synthesized. It contains amaleimide group (allowing coupling to the C-terminal sulphhydryl group of the fusion protein), an N-hydroxysulphosuccinimide group (allowing coupling to free amino groups of BPTI), and a photoreactive diazirine group. With this crosslinker, between 30% and 75% of the coxIV-DHFR fusion protein could be coupled to BPTI (see Figs below).

The photo-crosslinked product

When the chimaeric precursor bearing the trifunctional crosslinker was incubated with energized yeast mitochondria, ~40% of the added molecules were partially translocated into the organelles and processed by the matrix protease (Fig. 2, lane 2). After illumination of the mitochondria, 7–9% of the stuck chimaeric protein was converted to a species of apparent molecular mass 94K (Fig. 2, lane 3). This species was not formed if import of the DHFR domain was prevented by uncoupling the mitochondria (Fig. 2, lane 9). The photo-crosslink was thus specific for the cleaved, membrane-spanning precursor.

When mitochondria containing the stuck precursor were treated with 50 $\mu\text{g ml}^{-1}$ of proteinase K before the photoreaction, generation of the photoreaction product was partly

inhibited (Fig. 2, lane 5), suggesting that the protein participating in the crosslink was accessible to externally added protease. This inhibition was not caused by the removal of unprocessed precursor from the mitochondrial surface; when mitochondria were treated with proteinase K concentrations of 25–200 $\mu\text{g ml}^{-1}$, unprocessed precursor bound to the mitochondria was completely degraded at the lowest protease concentration, whereas the amount of the photoreaction product was significantly decreased only at higher protease concentrations (data not shown). These higher levels of protease, therefore, must have degraded the protein participating in the crosslink, indicating that this protein is exposed on the mitochondrial surface.

As the 94K reaction product was precipitated by affinity-purified anti-BPTI-antibodies, it contained the BPTI moiety

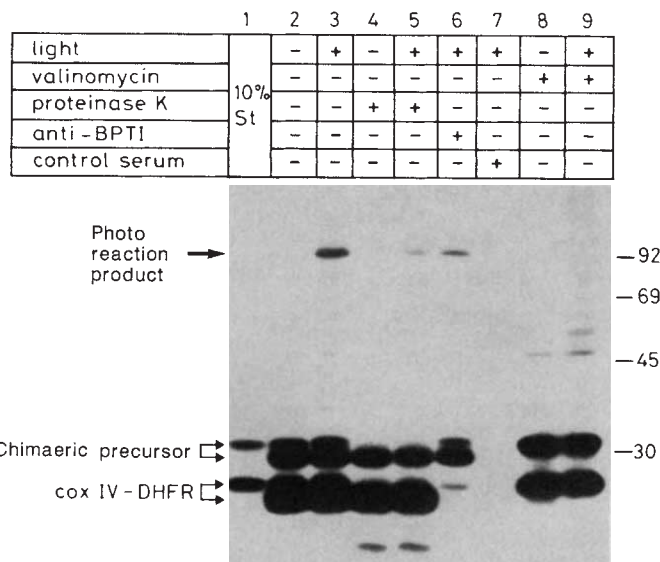


FIG. 2 The transmembrane chimaeric precursor photo-crosslinks to a single protein band. Lane 1, 10% of the amount of chimaeric precursor and residual underivatized coxIV-DHFR that was added to each import reaction. Lane 2, after import into mitochondria. Lane 3, after import followed by illumination of the mitochondria. Lanes 4 and 5, the same as lanes 2 and 3, respectively, except that, after completion of import, mitochondria were treated with 50 $\mu\text{g ml}^{-1}$ proteinase K for 30 min on ice, followed by addition of phenylmethylsulphonyl fluoride to 0.5 mM. Lanes 6 and 7, after import and illumination, 30 min on ice, followed by addition of phenylmethylsulphonyl fluoride to 0.5 mM. Lanes 6 and 7, after import and illumination, mitochondria were lysed with 0.5% Triton X100 and immunoprecipitated with affinity-purified anti-BPTI IgG (lane 6) or pre-immune IgG (lane 7). Lanes 8 and 9, the same as lanes 2 and 3, respectively, except that mitochondria were de-energized by 10 $\mu\text{g ml}^{-1}$ valinomycin before import. The positions of relative molecular mass (in K) markers are indicated on the right. Cleaved and uncleaved forms are indicated by arrows on the left for both precursor proteins.

METHODS. The chimaeric precursor was synthesized as described⁷, except that the trifunctional crosslinker shown in Fig. 1 was used (dissolved in 20 mM NaPi, pH 7.0) and the incubation time for BPTI with the crosslinker was extended to 45 min at room temperature. Unreacted crosslinker was removed by two successive gel filtration steps. Mitochondria were isolated as described¹². Import experiments were performed for 15 min at 30 °C with 150 μg (protein basis) of isolated yeast mitochondria in 230 μl import buffer¹³ with 40 μl (500 ng; 510⁵ d.p.m) of the mixture of chimaeric and underivatized precursor. Photoactivation was performed as described¹⁴. For immunoprecipitations, mitochondria were lysed in 100 μl 50 mM Tris-Cl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X100. The extract was preincubated with 400 μl of buffer A (50 mM Tris-Cl pH 8.5, 500 mM NaCl, 5 mM EDTA, 0.05% Triton X100, 1 mg ml^{-1} ovalbumin) and 60 μl of fixed *Staphylococcus aureus* cells (Pansorbin, Calbiochem) for 50 min on ice. After pelleting the *S. aureus* cells, the extract was incubated with 100 μg IgG for 2 h at 4 °C and then for 1 h with 110 μl *S. aureus* cells. The cells were pelleted and washed twice with buffer A and once with 20 mM Tris-Cl pH 8.0, 150 mM NaCl. Antigens were released by boiling in SDS-PAGE sample buffer and analysed by SDS-11% PAGE and fluorography of the dried gels.

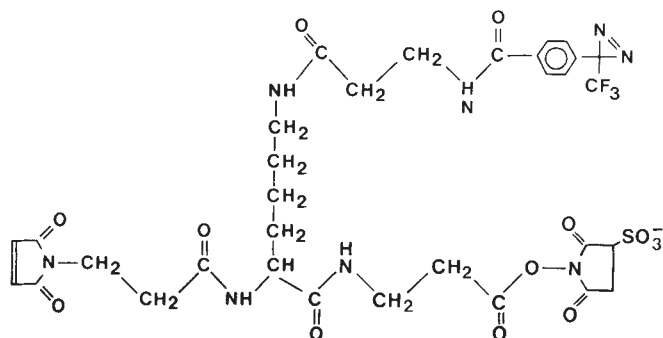


FIG. 1 The trifunctional photoreactive crosslinker. The maleimide- and N-hydroxy succinimide groups were used to conjugate BPTI to the precursor protein coxIV DHFR, positioning the photoreactive group between the two proteins.

METHODS. The crosslinker was synthesized by acylation of β -alanine with N-BOC-N-CBZ-L-lysine-N-hydroxysuccinimide ester (Bachem); removal of the N-CBZ group, followed by acylation of the free amino group with N-[4-[3-(trifluoromethyl)diaziriny]benzoyl]- β -alanine-N-hydroxysuccinimide ester; removal of the N-BOC group followed by acylation of the free amino group with 3-maleimido-propanoic acid-N-hydroxysuccinimide ester (Fluka); and formation of the active N-hydroxy-sulphosuccinimide ester¹¹.

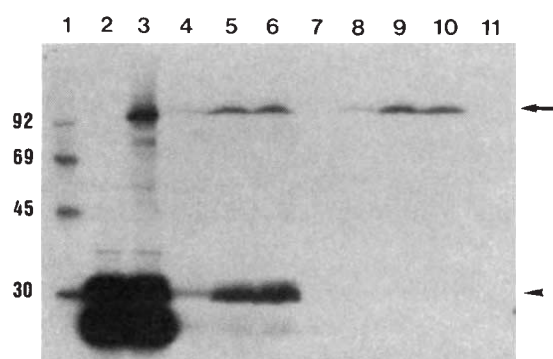


FIG. 3 Antisera against a partially purified 45K outer-membrane protein precipitate the photo-crosslinked product. Lane 1, ^{14}C -labelled molecular mass standards with sizes (K) given on the left; lanes 2 and 3, mitochondria after incubation with a mixture of the chimaeric and the underivatized fusion protein without (lane 2) and with (lane 3) subsequent illumination; lanes 4–11, as lane 3 except that, after illumination, mitochondria were solubilized in 1% Triton X100 (lanes 4–7) or in hot 5% SDS (lanes 8–11); the extracts were then incubated with three different rabbit antisera raised against partially purified 45K outer-membrane protein (lanes 4–6 and 8–10, respectively) or with non-immune serum (lanes 7 and 11). Note that the processed form of the stuck chimaeric precursor (arrowhead) is co-precipitated with the photo-crosslinked product after solubilization with Triton X100, but not after solubilization with hot SDS; by contrast, the photo-crosslinked product (arrow) is precipitated independently of the solubilization method used. **METHODS.** Import experiments and immune precipitations from Triton X100 extracts were done as described in Fig. 2. Alternatively, mitochondria (150 μg) were lysed in 50 μl 5% SDS, 50 mM Tris-Cl pH 8.0 for 5 min at 95 $^{\circ}\text{C}$; the extract was diluted with 1.65 ml of 50 mM Tris-Cl pH 8.0, 150 mM NaCl, 5 mM EDTA and 1% Triton X100. Preadsorption with Pansorbin and immunoprecipitations were performed as in Fig. 2. For each precipitation, 40 μl of rabbit serum and 200 μl of Pansorbin were used. The rabbit antisera had been raised against a partially purified preparation of 45K outer-membrane proteins; purification involved solubilization of the membranes with octylpolyoxyethylene followed by FPLC on MonoQ and MonoP columns (unpublished results).

(Fig. 2, lane 6). Because it was radioactive, it also contained the DHFR moiety (DHFR was the only radioactive moiety of the chimaeric precursor).

Partly translocated precursor

To identify the mitochondrial protein in the crosslinked product, we tested whether this product was immunoprecipitated by antisera against individual outer-membrane proteins. Of several sera tested, only antisera against a partially purified preparation of 45K proteins of the outer membrane immunoprecipitated the crosslinked product (Fig. 3, lanes 4–6 and 8–10). After solubilization of mitochondria with hot sodium dodecyl sulphate, only the photo-crosslinked product was precipitated (lanes 8–10); after solubilization with the non-ionic detergent Triton X100, however, underivatized, processed chimaeric precursor was co-immunoprecipitated with the crosslinked product (lanes 4–6). Association of the stuck precursor with components of the import machinery seems, therefore to survive solubilization with Triton X100 even in the absence of a covalent crosslink.

As shown previously⁴, the antisera we raised against 45K outer-membrane proteins are not monospecific; if tested by immune blotting against electrophoretically resolved outer membrane, they react with several proteins. Antibodies reacting with each of these proteins were, therefore, removed individually from the serum by preadsorbing aliquots with the corresponding protein band bound to nitrocellulose strips; the preadsorbed aliquots were then retested for their ability to immunoprecipitate the crosslinked product. This ability was abolished only upon removal of antibodies against a 42K protein (data not shown). To verify this result, IgG antibodies which had bound to either

the 45K protein band or the 42K protein were eluted from the nitrocellulose strips and tested for their ability to immunoprecipitate either outer-membrane proteins or the cross-linked product (Fig. 4a). IgG antibodies affinity-purified on the 45K protein band efficiently precipitated 45K outer-membrane proteins, but failed to recognize the crosslinked product (Fig. 4, lanes 2, 6); IgG antibodies affinity purified against the 42K protein precipitated this protein (as well as some 45K proteins) and also precipitated the crosslinked product (Fig. 4a, lanes 3, 7). The residual activity against 45K proteins was not surprising as the 42K band could not be completely separated from the adjacent, much more abundant 45K proteins. Antisera raised against the 45K band can thus be efficiently depleted of anti-42K antibodies, whereas the reverse is not true.

The presence of a 42K outer-membrane protein in the cross-linked product was further documented by the observation that

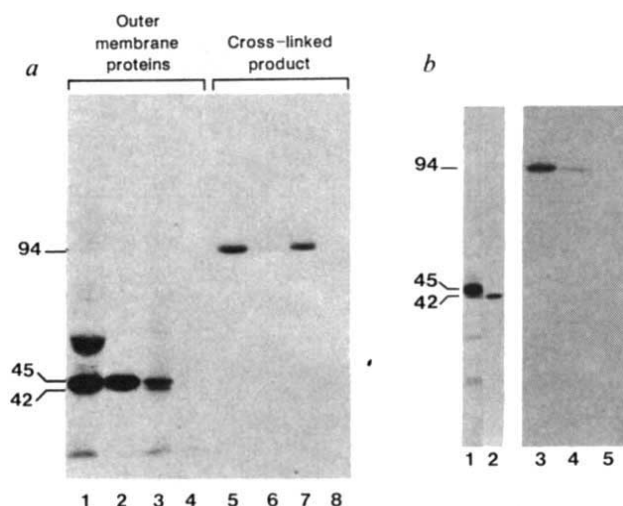


FIG. 4 The crosslinked product is immunoprecipitated by rabbit IgG affinity-purified against ISP 42K protein *a*, or by a monoclonal antibody against ISP 42 *b*. *a*, Lanes 1–4, immunoprecipitation of mitochondria labelled with [^{35}S]methionine *in vivo*; lanes 5–8, immunoprecipitation of unlabelled mitochondria that had accumulated the labelled photo-crosslinked 94K product. Lanes 1 and 5, 50 μg IgG isolated from an antiserum against partly purified 45K protein; lanes 2 and 6, 5 μg IgG affinity-purified against 45K proteins; lanes 3 and 7, 3 μg IgG affinity-purified against ISP 42; lanes 4 and 8, 8 μg IgG isolated from non-immune serum. *b*, Lanes 1 and 2, immunoblot of mitochondrial outer-membrane proteins with: lane 1, IgG from antiserum raised against partially purified 45K outer-membrane proteins; lane 2, anti-ISP 42 monoclonal antibody. Lanes 3–5, mitochondria were allowed to accumulate the photocrosslinked product, solubilized in SDS, and immunoprecipitated with: lane 3, 50 μg IgG against partly purified 45K outer-membrane proteins; lane 4, anti-ISP 42 monoclonal antibody (IgG from 25 ml culture supernatant); lane 5, a mock 'IgG' fraction from 25 ml culture supernatant obtained from a control hybridoma line. Molecular mass sizes (in K) are indicated on the left.

METHODS. Mitochondria were isolated from yeast cells labelled with [^{35}S]methionine as described¹⁵. Their specific radioactivity was 6×10^7 c.p.m. mg^{-1} protein. IgG antibodies were isolated from total serum by protein A-Sepharose chromatography¹⁶ or eluted from antigens immobilized on nitrocellulose strips with 0.1 M glycine/HCl pH 2.5 (3 min at 4 $^{\circ}\text{C}$), followed by neutralization to pH 8.0 and protein A-Sepharose chromatography. Import experiments were done as described in Fig. 2 and immunoprecipitations in the presence of SDS were performed as described in Fig. 3 except that for use with the monoclonal antibody Pansorbin was preloaded with rabbit anti-mouse immunoglobulin. The immunoblot experiments shown in *b* were done either with 5 μg of rabbit IgG per lane (followed by decoration with [^{125}I]labelled protein A) or with 1 ml of hybridoma culture supernatant, concentrated three-fold per lane (followed by sequential decoration with 10 μg ml^{-1} rabbit anti-mouse immunoglobulin and [^{125}I]labelled protein A). The monoclonal antibody (IgG₁ subclass) was prepared essentially as in 10. *a*, 8% polyacrylamide gel; *b*, 10% polyacrylamide gel. Molecular mass sizes (in K) are indicated on the left margins.

this product was immunoprecipitated by a monoclonal antibody which specifically recognized a 42 K outer-membrane protein (Fig. 4b). This antibody had been generated by injecting mice with right-side-out outer-membrane vesicles¹⁰. Although immunoprecipitation by this monoclonal antibody was not as efficient as that by rabbit antisera, it was specific (compare lanes 4 and 5, Fig. 4b). We conclude that the stuck chimaeric precursor is crosslinked to a 42K outer-membrane protein (ISP-42).

Antibodies against ISP 42

Antibodies raised against a major 45K band of outer membrane proteins inhibit protein import into mitochondria⁴. The results mentioned above prompted us to check whether this inhibition was caused by contaminating antibodies against the ISP 42. This was indeed the case. When the antiserum was depleted of IgG antibodies against 45K proteins (Fig. 5a) or against 30K or 60K proteins (data not shown), its inhibitory activity on protein import was not significantly decreased; removal of anti-ISP IgG antibodies, however, eliminated the inhibition. Conversely, IgG antibodies affinity-purified on immobilized ISP-42 inhibited

mitochondrial protein import, whereas IgG antibodies affinity-purified on immobilized 45K proteins were without effect (Fig. 5b). These results suggest strongly that ISP 42 is required for the import of precursor proteins into isolated mitochondria.

Conclusions

We show that a 42K protein of yeast mitochondria can be crosslinked to a precursor protein that is stuck in the protein-import machinery. The crosslink seems to reflect a specific association between these two proteins: it occurs with high efficiency and selectivity and only with partly translocated, but not with surface-bound precursor molecules. We conclude that ISP 42 is a component of the mitochondrial protein import site. This is further supported by the finding that IgG antibodies directed against ISP 42 block protein import into mitochondria.

ISP 42 is accessible to protease or IgG antibodies under conditions which preserve the integrity of the outer-membrane barrier. On subfractionation of mitochondria, it cofractionates with outer membranes without being enriched in membrane 'contact sites' (L. Pon and D. Vestweber, unpublished results). ISP 42 is thus exposed on the cytosolic face of the outer membrane.

Its location on the mitochondrial surface suggests that ISP 42 mediates one of the early steps of protein import. It may also be involved, however, in later steps as it is still closely associated with the partly translocated processed precursor.

When yeast mitochondrial outer membranes are fractionated by SDS-PAGE or by column chromatographic methods, ISP 42 is difficult to separate from the major 45K proteins (for example, see Fig. 4a, lane 3). As a result, all of our antisera raised against 45K proteins also contain anti-ISP 42 antibodies. This serendipity allowed us to track down the presence of ISP 42 in the crosslinked product. Initially we viewed ISP 42 as a proteolytic

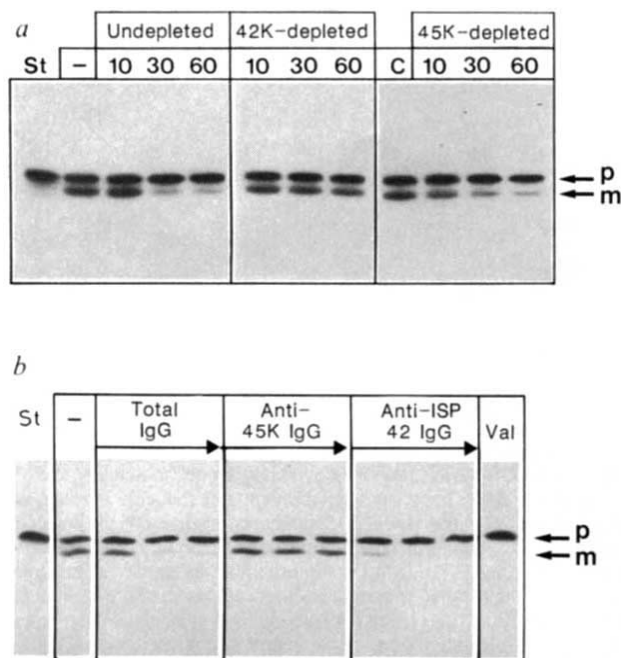


FIG. 5 IgG antibodies against the 42K protein inhibit protein import into isolated yeast mitochondria. *a*, Antiserum against partially purified 45K outer-membrane proteins was depleted of antibodies against either 45K proteins or ISP 42 by preadsorption with individual bands of these proteins immobilized on a nitrocellulose strip; IgG antibodies were isolated from these depleted aliquots as well as from an undepleted aliquot by protein A-Sepharose chromatography and tested for their ability to inhibit protein import by isolated yeast mitochondria. *b*, IgG antibodies from the undepleted anti-45K-protein serum as well as IgG affinity-purified from the immobilized bands of 45K outer-membrane proteins or ISP 42 (see above) were tested as in *a*. In *a*, the amount of IgG (in μ g) added to each mitochondrial sample is indicated on top of each lane; the lane marked '-' received no IgG. In *b*, these amounts (in μ g) were as follows: -, no IgG; Total IgG, 10, 40 and 80; Anti-45K IgG 1, 2 and 3; Anti-ISP 42 IgG 1, 2 and 3. Val, mitochondria de-energized by $10 \mu\text{g ml}^{-1}$ valinomycin. This negative control establishes that generation of the processed precursor is a valid measure of import. St, 20% of the fusion protein that was added to each mitochondrial sample. p and m, precursor and 'mature' form of the fusion protein.

METHODS. Yeast mitochondria ($10 \mu\text{g}$ protein) were incubated for 45 min at 0°C without or with the indicated amounts of IgG in 0.6 M sorbitol, 20 mM HEPES-potassium hydroxide pH 7.4, 50 mM glycine, 50 mM Tris (final volume, $40 \mu\text{l}$) and tested for import of purified labelled coxIV-DHFR fusion protein (6×10^4 c.p.m.; 12 min at 30°C). Samples were analysed by SDS-12% PAGE and fluorography.

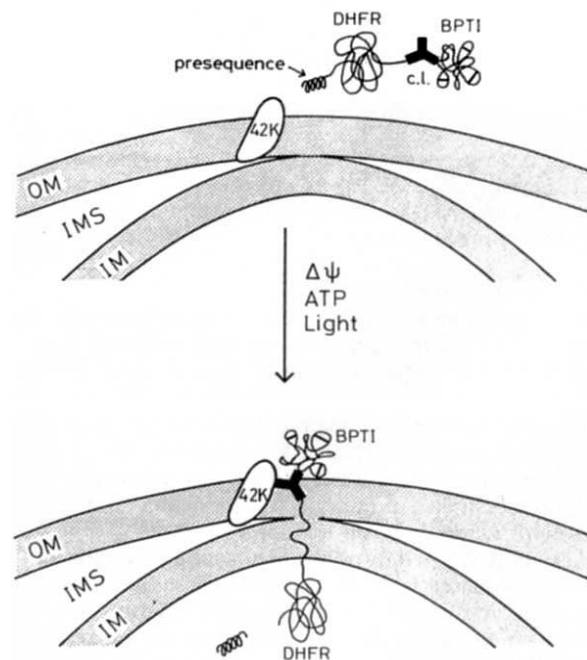


FIG. 6 The photo-crosslinking approach detects ISP 42 in the vicinity of a translocating protein. The coxIV-DHFR fusion protein coupled by a trifunctional crosslinker to BPTI is partly imported by isolated mitochondria in a reaction requiring ATP and an electrochemical potential across the inner membrane. The DHFR moiety of the partly translocated precursor is completely transferred into the matrix, the presequence is cleaved off, and the BPTI moiety remains accessible on the mitochondrial surface. The protein thus spans both membranes. Because it blocks protein import into mitochondria, it remains stuck in the protein import machinery⁷. Upon exposure to high-energy light, the photoreactive group in the trifunctional crosslinker covalently links the precursor protein to ISP 42, inner membrane; IMS, intermembrane space.

fragment of one of the 45K proteins⁴. The experiments reported here, however, show clearly that ISP 42 is a distinct protein and that the inhibition of protein import by antisera raised against the 45K protein band⁴ is caused by antibodies against ISP 42.

On SDS-polyacrylamide gel electrophoresis, the apparent molecular weight of the crosslinked product (94K) is larger than the sum of the two partners (32K and 42K). As the crosslinked product must have a branched, triskelion-like structure, its aberrant migration in SDS-polyacrylamide gels is explicable.

Received 19 July; accepted 29 August 1989.

- Attardi, G. & Schatz, G. A. *Rev. Cell Biol.* **4**, 289–333 (1988).
- Riezman, H., Hay, R., Witts, C., Nelson, N. & Schatz, G. *EMBO J.* **2**, 1113–1118 (1983).
- Zwizinski, C., Schleyer, M. & Neupert, W. *J. biol. Chem.* **259**, 7850–7856 (1984).
- Ohba, M. & Schatz, G. *EMBO J.* **6**, 2109–2115 (1987).
- Gillespie, L. L. *J. biol. Chem.* **262**, 7939–7942 (1987).
- Pain, D., Kanwar, Y. S. & Blobel, G. *Nature* **331**, 232–237 (1988).
- Vestweber, D. & Schatz, G. *J. Cell Biol.* **107**, 2037–2043 (1988).
- Hurt, E. C., Pesold-Hurt, B. & Schatz, G. *FEBS Lett.* **178**, 306–310 (1984).
- Pon, L., Moll, T., Vestweber, D., Marshall, B. & Schatz, G. *J. Cell Biol.* (in the press).

ISP 42 is probably not the only protein mediating the import of precursor proteins into mitochondria. The 30K polypeptides identified in mammalian mitochondria⁵ and chloroplasts⁶ may represent an additional component of the import machinery in these two organelles. Characterization of a multi-component system is usually quite rapid once the first component has been identified. The results presented here should thus help in detecting additional proteins of the mitochondrial protein-import system. □

- Riezman, H. *et al. EMBO J.* **2**, 1105–111 (1983).
- Staros, J. V. *Biochemistry* **21**, 3950–3955 (1982).
- Daum, G., Boehni, P. C. & Schatz, G. *J. biol. Chem.* **257**, 13028–13033 (1982).
- Gasser, S. M., Daum, G. & Schatz, G. *J. biol. Chem.* **257**, 13034–13041 (1982).
- Meister, H., Bachofen, R., Semenza, G. & Brunner, J. *J. biol. Chem.* **260**, 16326–16331 (1985).
- Reid, G. A. *Meth. Enzym.* **97**, 324–329 (1983).
- Bigbee, W. L., Vanderlaan, M., Fong, S. S. N. & Jensen, R. H. *Molec. Immun.* **20**, 1353 (1983).

ACKNOWLEDGEMENTS. This study was supported by the Swiss National Science Foundation (G.S. and J.B.), the US Public Health Service (G.S.) and EMBO (D.V. and A.B.).

Structure of the guanine-nucleotide-binding domain of the Ha-ras oncogene product p21 in the triphosphate conformation

Emil F. Pai, Wolfgang Kabsch, Ute Krengel, Kenneth C. Holmes, Jacob John & Alfred Wittinghofer

Max-Planck-Institut für medizinische Forschung, Abteilung Biophysik, Jahnstrasse 29, 6900 Heidelberg, FRG

The crystal structure of the guanine-nucleotide-binding domain of p21 (amino acids 1–166) complexed to the guanosine triphosphate analogue guanosine-5'-(β , γ -imido)triphosphate (GppNp) has been determined at a resolution of 2.6 Å. The topological order of secondary structure elements is the same as that of the guanine-nucleotide-binding domain of bacterial elongation factor EF-Tu. Many interactions between nucleotide and protein have been identified. The effects of point mutations and the conservation of amino-acid sequence in the guanine-nucleotide-binding proteins are discussed.

GUANINE-nucleotide-binding proteins, such as elongation factors and signal-transducing G proteins, exert their function by cycling between an inactive, GDP-containing and an active, GTP-containing conformation^{1–3}. The cycling between these conformations is mediated by exchange factors or hormone-activated receptors which increase the exchange rate of the strongly bound GDP with GTP. The system returns to the ground state by the intrinsic GTPase activity of the protein. The GTPase reaction is usually increased by the effector, which in turn is switched on by the interaction.

The product of the N-, Ki- and Ha-ras genes, p21, is a small guanine-nucleotide-binding protein that has been found to be mutated in a large number of human tumours⁴. Its exact cellular role is unknown, although it is believed to be involved in a growth-promoting signal-transduction pathway. As with all other guanine-nucleotide-binding proteins, p21 is active only in

the GTP-bound form; only in this form does it interact with GAP (GTPase activating protein) which is either an effector of ras action or its negative regulator^{5–7}. The interaction between p21 and GAP ensures that p21 is maintained in the GDP bound conformation.

The crystal structure of the p21-GDP complex has recently been described⁸. We were able to crystallize the guanine-nucleotide-binding domain of p21^{Ha-ras} in the active GTP conformation by using various slowly hydrolysing GTP analogues⁹. We report the three-dimensional structure of the guanosine-5'-(β , γ -imido)-triphosphate (GppNp) complex determined by X-ray crystallography. This should enable us to define the conformational transition between the GDP and the GTP bound states and to investigate in detail the site of interaction of p21 with GAP.

Our chain tracing is different from that reported earlier for the p21-GDP complex. After communicating our findings to Kim and coworkers, they reevaluated their interpretation. Their revised chain tracing, as described in correspondence to *Science*¹⁰, now agrees with that presented here.

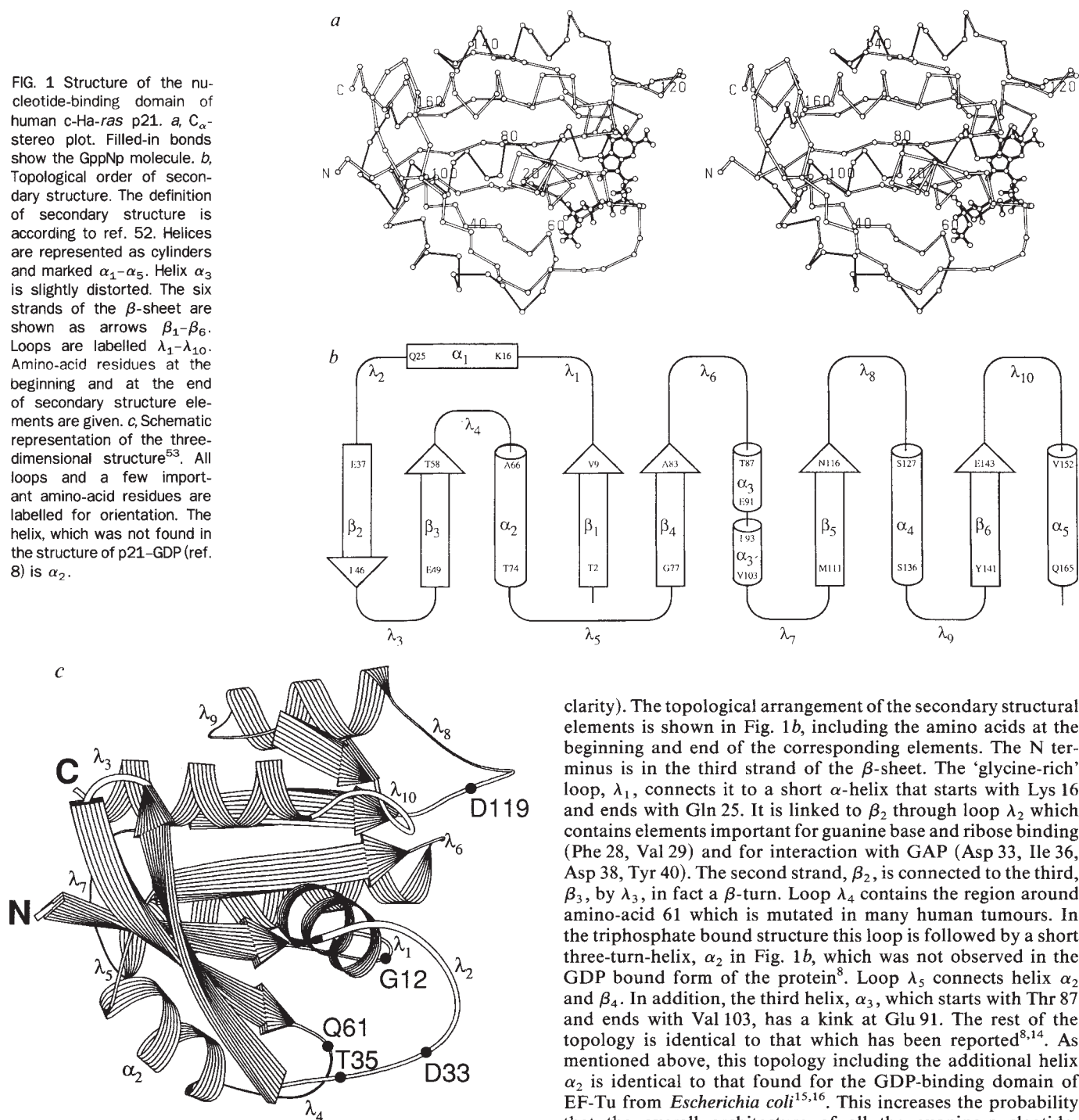
Protein preparation and crystallization

Cloning, over-expression and purification of the truncated form p21_c (amino acids 1–166) was performed as described for the native protein^{11,12}. C-terminal truncation did not affect nucleotide binding or GTPase activity¹³. Crystals of the complex of p21_c and the slowly hydrolysing analogue GppNp were grown according to Scherer *et al.*⁹.

Backbone structure

In the final stages of our model building it became obvious that we would not be able to obtain a good fit between model and observed electron density, when following the chain tracing as reported by DeVos *et al.*^{8,14}, for the GDP complex. The largest discrepancies arose at Tyr 4 where there was not sufficient density to account for the aromatic ring and in the connecting

FIG. 1 Structure of the nucleotide-binding domain of human c-Ha-ras p21. *a*, C_{α} -stereo plot. Filled-in bonds show the GppNp molecule. *b*, Topological order of secondary structure. The definition of secondary structure is according to ref. 52. Helices are represented as cylinders and marked α_1 – α_5 . Helix α_3 is slightly distorted. The six strands of the β -sheet are shown as arrows β_1 – β_6 . Loops are labelled λ_1 – λ_{10} . Amino-acid residues at the beginning and at the end of secondary structure elements are given. *c*, Schematic representation of the three-dimensional structure⁵³. All loops and a few important amino-acid residues are labelled for orientation. The helix, which was not found in the structure of p21-GDP (ref. 8) is α_2 .



clarity). The topological arrangement of the secondary structural elements is shown in Fig. 1*b*, including the amino acids at the beginning and end of the corresponding elements. The N terminus is in the third strand of the β -sheet. The 'glycine-rich' loop, λ_1 , connects it to a short α -helix that starts with Lys 16 and ends with Gln 25. It is linked to β_2 through loop λ_2 which contains elements important for guanine base and ribose binding (Phe 28, Val 29) and for interaction with GAP (Asp 33, Ile 36, Asp 38, Tyr 40). The second strand, β_2 , is connected to the third, β_3 , by λ_3 , in fact a β -turn. Loop λ_4 contains the region around amino-acid 61 which is mutated in many human tumours. In the triphosphate bound structure this loop is followed by a short three-turn-helix, α_2 in Fig. 1*b*, which was not observed in the GDP bound form of the protein⁸. Loop λ_5 connects helix α_2 and β_4 . In addition, the third helix, α_3 , which starts with Thr 87 and ends with Val 103, has a kink at Glu 91. The rest of the topology is identical to that which has been reported^{8,14}. As mentioned above, this topology including the additional helix α_2 is identical to that found for the GDP-binding domain of EF-Tu from *Escherichia coli*^{15,16}. This increases the probability that the overall architecture of all the guanine-nucleotide-binding proteins is very similar and that perhaps the same structural elements are involved in their transition between GDP- and GTP-bound forms.

Figure 1*c* shows the p21_c-GppNp structure as a ribbon drawing. All loops and helix α_2 not present in the GDP form⁸ are labelled. Loop λ_2 contains the site of interaction with the possible effector molecule GAP (ref. 6). It is highly exposed to the solvent and is probably in a different conformation from that found in the GDP bound structure, as p21 in the GDP bound form only weakly interacts with GAP (ref. 6). The sites of oncogenic activation on loops λ_1 and λ_4 , and some selected amino acids, are indicated.

The phosphate binding loop

Many mononucleotide-binding proteins have a stretch of amino acids with the consensus sequence G-X-X-X-G-K-S/T

loops, especially in the 'glycine-rich' loop (residues 10–16) where gaps in the electron density had to be crossed. When β -strands 3 and 1 in Fig. 3 of ref. 8 were changed to β_1 and β_3 , respectively, and the connecting loops corrected accordingly (Fig. 1*b*, *c*), these problems could be completely resolved. With this chain tracing there is a continuous arrangement of main chain atoms in the electron density. It should be noted that our interpretation is consistent with p21 having the same topology as the GDP-binding domain of EF-Tu, the only other guanine-nucleotide-binding protein with a known three-dimensional structure^{15,16}. It also confirms the structural model of p21 (refs 16–18) which was based on the sequence homology between EF-Tu and p21 (ref. 19).

Figure 1*a* shows a C_{α} stereo plot of the nucleotide-binding domain of the p21 protein together with an atomic structure of GppNp (calculated positions of some hydrogens are added for

TABLE 1 Statistics of data collection

	Native	MMN	K ₂ [Hg(CN) ₄]	K[Au(CN) ₂]
Heavy atom derivatives	—	Co-crystallized, molar ratio 1:2	Soaking for 14 h, concentration 2 mM	Co-crystallized, molar ratio 1:3
Resolution (Å)	2.6	2.8	2.6	3.0
Observed reflection (no.)	24,377	11,871	12,550	6,496
Unique reflection (no.)	5,378	3,778	5,299	3,194
R _{sym} (%) *	5.2	6.0	4.7	10.3
R _F (%) †	—	21.5	32.1	16.5

All X-ray diffraction data from the crystals were recorded on an electronic area detector (Siemens/Nicolet, Madison, WI). X-rays (CuK_α) were generated using a GX-18 rotating anode (Elliot/Enraf-Nonius, Delft) and focused by Franks double-mirror optics. The anode was operated as 35 kV and 50 mA. An oscillation camera (Supper, Natick, Mass.) with a vertical rotation axis was used to move the crystal. Camera and detector were controlled by a simplified version of a program by Durbin *et al.*⁴⁵ running on a small dedicated computer (PCS, München, FRG). For each data-collection run a sequence of adjacent electronic rotation pictures (data frames) was transferred by Ethernet to a large computer (CONVEX C210) and processed by the program XDS (refs 46, 47). The program terminates with a list of squared structure factor amplitudes corrected for absorption and radiation damage. Only one heavy-atom derivative (K₂[Hg(CN)₄]) could be obtained using the soaking technique. Two more derivatives were prepared by co-crystallizing the protein with heavy atoms. Each data set was obtained from one crystal to avoid systematic errors in heavy-atom occupancies for the derivative crystals. Two data sets were collected for a native crystal by changing the orientation of the capillary after the first set. The two sets were processed independently and subsequently scaled together. The symmetry R factor in intensities for all reflections was 5.2%, or 4.1% and 4.3% for the two separate runs, respectively. MMN, CH₃HgNO₃.

* $R_{\text{sym}} = \sum_h \sum_i |I_{hi} - \bar{I}_h| / \sum_h \sum_i I_{hi}$ where I_{hi} are symmetry related intensity observations and $\bar{I}_h$ is the mean intensity of reflections with unique indices h .

† $R_F = 2 \sum_h |F_{\text{PH}} - F_P| / \sum_h |F_P + F_{\text{PH}}|$ where F_{PH} and F_P are the derivative and native structure factor amplitudes, respectively.

(single-letter amino-acid code) which connects a β -strand with an α -helix. This loop usually contains additional glycines or prolines. In p21 the sequence of this element is G₁₀-A-G-G-V-G-K-S₁₇ and the mutation of Gly 12 to any other amino acid except proline activates the transforming potential of the protein. This is also true for the replacement of Gly 13 by valine. Figures 2 and 3a show the arrangement of the corresponding residues in the triphosphate-bound structure of p21. The three main-chain nitrogens of residues 13 to 16 in the loop and those of residues 17 and 18 in the following α -helix, point towards and are within 3.5 Å of the phosphate oxygens and are thus able to form hydrogen bonds. At the present resolution, the amide nitrogen of residue 16 is closest (3.0 Å) to one of the β -phosphate oxygens. The amide nitrogen of residue 18, which is located in helix α_1 following loop λ_1 , forms a hydrogen bond to the pro-R oxygen of the α -phosphate. This is in agreement with our results obtained with α -thiophosphate analogues of GDP and GTP, which show that the replacement of the pro-S oxygen by sulphur is better tolerated than the replacement of the pro-R oxygen^{11,20}.

Lys 16 stretches across the phosphates. Its amino group is close enough to the γ -phosphate for an ion-pair interaction and forms hydrogen bonds with the main-chain oxygens of residues 10 and 11. It is remarkable that the same structural arrangement of lysine has been reported for the nucleotide-binding loop of porcine adenylate kinase, an ATP-binding protein. Here too, the equivalent lysine residue (Lys 21) makes contact with the

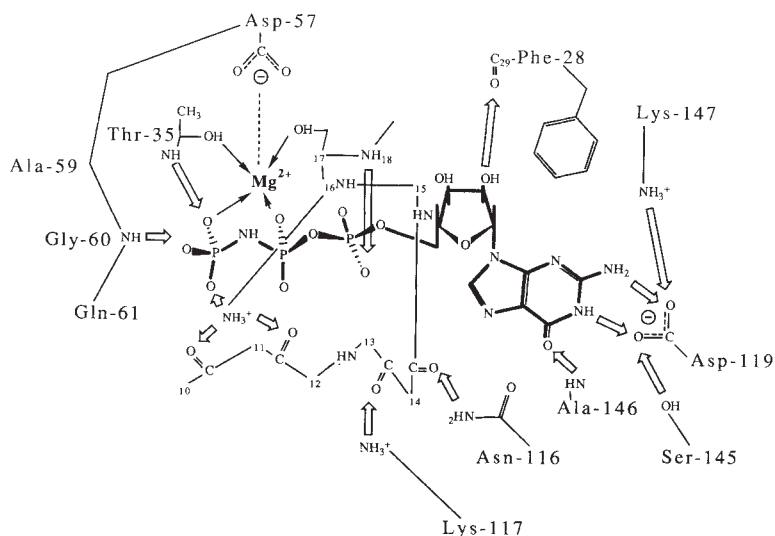
main chain at the corresponding positions (Gly 15, Gly 16) (ref. 21). Point mutations in the nucleotide-binding loop of *E. coli* adenylate kinase also have large effects on the kinetics and structure of the enzyme²², as has been found in p21.

The phosphate-binding loop has sometimes been called the flexible loop because of its high content of glycine residues. In the structure of the triphosphate conformation of p21, this region shows high density with low-temperature factors for all atoms. Taken together with the fact that many main chain nitrogen and oxygen atoms interact not only with the nucleotide, but also with other parts of the structure, this seems to indicate that the loop is very rigid (Fig. 3a).

Guanine nucleotide binding site

Details of the interactions between nucleotide and protein are shown in a sketch in Fig. 2. The guanine base is sandwiched between the aromatic side chain of Phe 28 on one side and the aliphatic part of the side chain of Lys 117 on the other. The amino group of Lys 117 is 3.5 Å away from the endocyclic oxygen of the ribose. It is close enough to the main chain carbonyl group of Gly 13 in the phosphate binding loop λ_1 to form a hydrogen bond and thereby connecting loops λ_8 and λ_1 . The strong hydrogen bond between O6 of the guanine ring and the amide nitrogen of Ala 146 is also important for the binding of the guanine base. It has been found²³, using an *in situ* colony hybridization assay, that the mutation Thr 144 → Ile reduces the

FIG. 2 Schematic drawing of protein-nucleotide interactions. Hydrogen bonds are indicated by open arrows ($\Rightarrow$), bonds between the magnesium ion and its ligands are shown as solid arrows ($\rightarrow$). Some important interactions between side-chain atoms are also included.



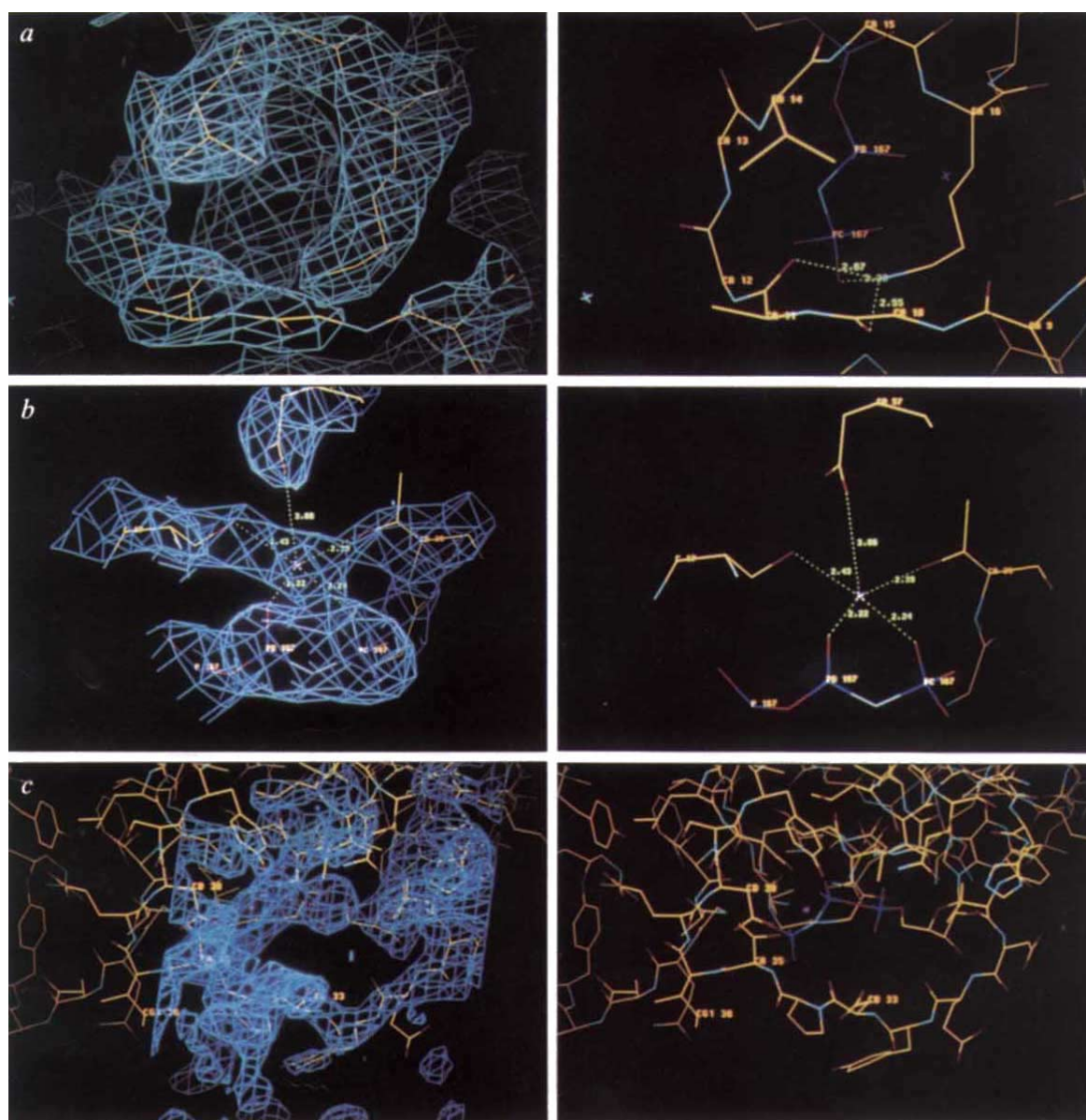


FIG. 3 Part of the 2.6 Å electron density map with the fitted molecular model. *a*, Loop λ_1 , the glycine-rich, phosphate-binding loop. All parts of this loop are in well defined density. There are no indications of flexibility. The side chain of Lys 16 folds back towards amino acids 10 and 11. It interacts with their backbone carbonyls and with the γ -phosphate. Electron density is contoured at 20% of maximum density. *b*, Magnesium ion surrounded by

its ligands. β - and γ -phosphate, Ser 17 and Thr 35 coordinate in an only slightly distorted planar configuration. Asp 57, which is a little further away, probably interacts with the metal ion via a water molecule. (Contour at 25%.) *c*, Loop λ_2 , which interacts with the GTPase activating protein, GAP. This stretch of residues is somewhat detached from the rest of the molecule making it easy for another protein to bind. (Contour at 20%.)

affinity for GDP. Our analysis suggests that this effect is an indirect one, through the local main-chain conformation, possibly weakening or even destroying the interaction of Ala 146 with O6 of GTP. We have also shown that h6-GDP, where the oxygen atom at position 6 of the guanine base has been removed, binds with 25-fold-lower affinity than GDP (Linke, R. *et al.*, unpublished results). Pointing to the importance of this interaction is the fact that a similar result with even lower relative affinity for h6-GDP has been found with EF-Tu (ref. 24).

Asp 119 is part of the N-K-X-D motif which is highly conserved in all guanine-nucleotide-binding proteins and is called the guanine-specificity region. The carboxylate group of Asp 119 contacts the exocyclic 2-amino group and the endocyclic N1 of guanine. This bonding scheme has been observed by NMR for the interaction between guanine- and a glutamic-acid containing dipeptide²⁵. Asp 119 is also close enough to interact with the

side chain hydroxyl of Ser 145 and with the amino group of Lys 147, both of which again are conserved in the small guanine-nucleotide-binding proteins. It is therefore, not surprising that the mutation of Asp 119 in p21 and of Asp 138 in EF-Tu drastically reduces the affinity for guanine nucleotides²⁶⁻³⁰.

The ribose of GppNp is in a 2'-endo conformation. Asp 30 is pointing in its direction but is too far away to make any hydrogen bonds. The main-chain carbonyl atom of residue 29, however, is within hydrogen bonding distance of the 2'-hydroxyl group of the ribose (Fig. 2). The 2'- and 3'-hydroxyl groups are exposed to the solvent, as one would expect, as at least one of the ribose hydroxyl groups of GTP or GDP can be substituted by the rather bulky fluorescent N-methylanthraniloyl group without causing a significant decrease in binding affinity³¹.

As in the p21-GDP structure, the phosphate-binding loop is wrapped around the β -phosphate group, with four main-chain

TABLE 2 Heavy-atom positions and refinement

<i>a</i> Positions		Fractional coordinates			Occupancy	Temperature		
Compound		<i>x</i>	<i>y</i>	<i>z</i>	factor			
MMN		0.9536	0.7773	0.0717	1.8036	2.9		
		0.3695	0.5037	0.0849	0.5573	6.3		
K ₂ [Hg(CN) ₄]		0.3613	0.5075	0.0823	2.1256	3.5		
		0.8228	0.4425	0.0316	1.6901	16.9		
K[Au(CN) ₂]		0.9439	0.7707	0.0695	0.4751	5.0		
		0.9495	0.8080	0.0726	1.1657	3.2		
<i>b</i> Refinement								
	Resolution (Å)	100–6.0	6.0–4.5	4.5–3.5	3.5–3.0	3.0–2.7	2.7–2.6	Total
	<i>⟨m⟩</i> *	0.77	0.71	0.64	0.57	0.55	0.60	0.63
MMN	<i>R</i> _C †	30.1	35.8	45.9	52.9	49.2	—	41.8
	<i>F</i> _H / <i>E</i> ‡	3.1	2.6	2.0	1.9	2.0	—	2.2
K ₂ [Hg(CN) ₄]	<i>R</i> _C	43.3	64.8	66.0	71.4	57.6	52.5	60.4
	<i>F</i> _H / <i>E</i>	2.2	1.4	1.1	1.1	1.3	1.5	1.4
K[Au(CN) ₂]	<i>R</i> _C	51.3	59.9	64.0	71.4	—	—	60.0
	<i>F</i> _H / <i>E</i>	1.8	1.5	1.3	1.1	—	—	1.4

The difference Patterson map for the CH₃HgNO₃ (MMN) derivative clearly revealed a single dominant heavy-atom site. All other derivatives were analysed by the difference Fourier technique using the phases derived from the MMN derivative. Six strands of β -sheet and four α -helices were identified in the initial MIR electron-density map. The loop regions, however, were not easy to interpret and some uncertainties about the correct connections remained. Omitting the dubious regions in the map, a polyaniline backbone model was fitted to the electron density using the interactive graphics program FRODO (ref. 48) adapted to an IRIS 4GT (Silicon Graphics Inc., Mountain View, Ca) by C. M. Cambillau. The partial model was refined to an R -factor of ~38% using the program PROLSQ (ref. 49). A new map was then calculated combining phase probability distributions from the partial model with the derivative information. Model bias was greatly reduced by incorporating the method developed by Read⁵⁰ into our program package. The new map was of better quality and most of the amino-acid side chains were clearly recognizable. The resulting new model was refined with the program XPLOR (ref. 51) to an R -factor of 33%. An additional helix (helix α_2 in Fig. 1*b*) was identified and two strands of β -sheet and three connecting loops were reassigned in the new map. Two more rounds of minor rebuilding and refinement were carried out. The last refinement was performed with the program PROLSQ and individual atomic temperature factors were determined. At this stage the R -factor dropped to 22.9% with all observed data from 10 to 2.6 Å resolution included in the refinement.

* $\langle m \rangle$ = mean figure of merit,

† $R_C = \sum |F_{\text{PH,obs}} - F_{\text{PH,calc}}| / \sum |F_{\text{PH,obs}} + F_{\text{PH,calc}}|$ for centric reflection

‡ $F_H/E = \sqrt{\sum f_H^2 / \sum (F_{\text{PH,obs}} - F_{\text{PH,calc}})^2}$

peptide nitrogens in the loop pointing towards the phosphates. Other main-chain amide nitrogens which interact with the phosphates are NH-17, NH-18, and that of Gly 60, located just after the end of β_3 and beginning of loop λ_4 . Gly 60 is conserved as part of the D-X-X-G motif in all guanine-nucleotide-binding proteins. Mutation of this residue in the α -chain of the G protein G_s creates a protein with equal affinity for GDP and GTP, which is, however, no longer able to stimulate adenylyl cyclase³². Also, the protein can no longer switch between the GDP and the GTP bound state³². This points to a structural role for this glycine residue in the guanine-nucleotide-binding proteins. In the structure presented here, the main chain at Gly 60 adopts a sharp bend not possible for any other amino acid.

Magnesium-ion coordination

Numerous studies of elongation factors, G proteins and p21 have shown that Mg²⁺ is essential for proper binding of ligands, for the GTPase activity and for the structural integrity of the proteins. We have shown before that the metal ion in the p21-bound GDP-Mg²⁺ has a monodentate coordination to the β -phosphate²⁰. In analogy to EF-Tu and other nucleotide-binding proteins, one would expect that p21 in the GTP complex binds the metal ion as a bidentate β - γ -complex³³. We have identified an electron density close to the β - and γ -phosphate groups as Mg²⁺, as excess Mg²⁺ is always present during protein purification and crystallization.

Magnesium ions in nucleotide-binding proteins have six ligands coordinated in an octahedral arrangement. Figures 2 and 3*b* show the coordination of Mg²⁺ in the triphosphate conformation of p21. In addition to the β - and γ -phosphates, we can identify at least two if not three ligands which are close enough to the metal ion to be in the first coordination sphere. These are Ser 17, Thr 35, and possibly Asp 57, all three of which are totally conserved in small guanine-nucleotide-binding proteins and the elongation factors (Palme, K. *et al.*, unpublished

results; Chardin, P., unpublished results). Ser 17 and Asp 57 are also conserved in the large G_α proteins. Although Ser 17 and Thr 35 are within bonding distance of the metal ion, Asp 57 points towards the metal ion but is so far away that there is possibly an intermediate water molecule. Figure 2 shows that Thr 35, in addition, interacts through the main-chain amide nitrogen with one of the γ -phosphate oxygens.

Structure of the effector loop

It has been shown previously that the site of interaction of GAP with p21 embraces amino acids 32 to 40. Certain mutations in this region abolish the ability of p21-GTP to be activated by GAP as well as its biological activity in yeast^{34–37}. Figure 1*b* shows that only amino acids 32–36 are situated in loop λ_2 . The other amino acids that are thought to be involved in GAP interaction are part of the second β -strand, β_2 . Figure 3*c* shows the three-dimensional structure of this region. The loop is detached from the otherwise compact structure of the molecule and would thus be easily accessible to other proteins. The residues which have specifically been shown by mutagenesis to be involved in the interaction with GAP are Asp 33, Thr 35, Ile 36, Asp 38 and Tyr 40 (refs 34–37). As described above, Thr 35 coordinates to Mg²⁺ and to the γ -phosphate. The effect of the Thr 35 → Ser or the more severe Thr 35 → Ala mutation on GAP activity therefore seems to be mediated by the manner in which these mutations change metal-ion binding. Figure 3*c* shows that the other residues in this area, believed to be involved in p21-GTP-GAP interaction, are more or less exposed to the solvent. The loop part of the effector region, amino acids 32–36, forms a ridge that sticks out of the rest of the protein. GAP interacts with p21 much more strongly in the GTP bound form than in the GDP bound form^{6,37} just as EF-Tu has a 100-fold higher affinity for aminoacyl-tRNA in the GTP complex³⁸. It is to be expected, therefore, that the structure of the effector loop is different in the two conformations.

Antibody Y13-259 binding site

The antibody Y13-259 (ref. 39) has been found to be of particular importance because its microinjection into NIH 3T3 cells and certain other fibroblastic cell lines causes reversion of the transformed phenotype induced by oncogenic *ras* mutations. The binding epitope of this antibody has been mapped by deletion cloning⁴⁰ and more precisely by site directed mutagenesis²⁸ to the region of p21 comprising amino acids 63–73. We find a new helix, α_2 , that comprises residues Ala 66 to Thr 74 (Fig. 1b) and was not found in the p21-GDP conformation⁸. Residues Ser 65, Ala 66, Met 67, Gln 70 and Arg 73 are on the outside of this helix α_2 facing the solvent. Such a helix had been predicted³⁷ on the basis of mutagenesis experiments, taking into account the sequence homology between EF-Tu and p21.

Oncogenic activation

Mutations in p21 amino-acid positions 12/13 and 61 have been found in a high percentage of human tumours. Additional activating mutations have been generated by site-directed or random *in vitro* mutagenesis (see ref. 4). Our three-dimensional structure shows that all of these mutants have substitutions of amino acids in regions found to be important for the binding of the guanine nucleotide. Biochemically, these mutations have an effect on the binding affinity of GDP/GTP, and/or the rate of *in vitro* GTPase activity, and/or the interaction with the GTP-activating protein GAP. It appears that at positions 12 or 13 only glycines can be tolerated because of the close spatial interaction between this loop, the phosphate residues, the effector loop λ_2 and the loop containing Gln 61. Any amino-acid substitution in the phosphate binding loop will effect the conformations of loops λ_2 and λ_4 .

Gln 61, with its large functional side chain, is at the beginning of a stretch of three or four amino acids which in our structure are less well defined than the rest of the chain, suggesting a higher mobility of these residues even in the crystal. Gln 61 is close to the γ -phosphate and one could envisage that replacing it with a hydrophobic side chain could destabilize a possible interaction with the phosphate group and/or the amino acids in its vicinity. It has indeed been found that mutations Gln 61 $\rightarrow$ Val and Gln 61 $\rightarrow$ Leu have the highest transforming efficiency of all Ha-*ras* mutants at this position^{41–42}.

The mutations in position 12 and 61 have large opposite effects on the nucleotide dissociation rate constants of p21. Val 12, Asp 12 and Arg 12 mutants have decreased, whereas Leu 61 has

drastically increased nucleotide dissociation rates. Also, the two kinds of mutants have opposite effects on the interaction with GAP. Gly 12 $\rightarrow$ Val as well as Gln 61 $\rightarrow$ Leu are able to bind to but are not activated by GAP (ref. 6). Gly 12 $\rightarrow$ Val has a lower affinity to GAP than wild type whereas Gln 61 $\rightarrow$ Leu has a higher affinity. Amino acids Gly 12 and Gln 61, which are the residues most often mutated in human tumours, are in fact in close proximity in the three-dimensional structure of the p21_c-GppNp complex (distance between C α s is 5.7 Å). The oncogenic activation would therefore effect the same region of the protein.

GTPase activity

It has been shown that GTP complexed to p21 or EF-Tu, is hydrolysed by direct attack from water with inversion of configuration at the γ -phosphate⁴³. With the current resolution of our analysis, we cannot identify with certainty any water molecule in a position to attack the γ -phosphate. As the rate of hydrolysis, albeit slow, is at least several hundred times faster than the hydrolysis of GTP in water, functional groups could be activating the water molecule or the phosphates or both.

According to analysis so far, only Lys 16 or Gln 61 would be located close enough for this role. Changing Ala 59 to a threonine would place its side-chain hydroxyl in a position to accept the γ -phosphate in the autophosphorylation reaction observed in viral p21 Ha-*ras* (ref. 12).

GAP stimulated hydrolysis by p21 is much faster than its intrinsic GTPase activity, probably as fast as that of G α proteins. Therefore, GAP may change the position of residues on p21 such that they can catalyse a fast GTP hydrolysis. Another possibility would be that GAP itself supplies the amino acids needed for fast hydrolysis. Figure 3c shows that the binding site of GAP is very open to the solvent at two regions that are on either side of the effector loop. There appears to be ample space for close contact between the two proteins around this region of the molecule.

We are currently working on the 1.5 Å resolution structure of the p21_c-GppNp complex. We hope to be able to analyse the GTPase reaction itself in more detail from crystals of a complex between p21 and a caged GTP analogue. These crystals are catalytically active after photoactivation and the GTPase reaction can be followed by Laue techniques using synchrotron radiation (ref. 44 and work in progress). Atomic coordinates will be deposited with the Protein Data Bank when refinement at 1.5 Å resolution has been completed. □

Received 11 July; accepted 25 August 1989.

- Kaziro, Y. *Biochim. biophys. Acta* **505**, 95–127 (1978).
- Gilman, A. G. *A. Rev. Biochem.* **56**, 615–649 (1987).
- Stryer, L. & Bourne, H. R. *A. Rev. cell Biol.* **2**, 391–419 (1986).
- Barbacid, M. *A. Rev. Biochem.* **56**, 779–827 (1987).
- Trahey, M. & McCormick, F. *Science* **238**, 542–545 (1987).
- Vogel, U. *et al. Nature* **335**, 90–93 (1988).
- Trahey, M. *et al. Science* **242**, 1697–1700 (1988).
- DeVos, A. *et al. Science* **239**, 888–893 (1988).
- Scherer, A. *et al. J. molec. Biol.* **206**, 257–259 (1989).
- Tong, L., Milburn, M. V., DeVos, A. M. & Kim, S. H. *Science* **245**, 244 (1989).
- Tucker, J. *et al. EMBO J.* **5**, 1351–1358 (1986).
- John, J., Frech, M. & Wittinghofer, A. *J. biol. Chem.* **263**, 11792–11799 (1988).
- John, J., Schlichting, I., Schiltz, E., Rösch, P. & Wittinghofer, A. *J. biol. Chem.* **264**, 13086–13092 (1989).
- Tong, L. *et al. Nature* **337**, 90–93 (1989).
- LaCour, T. F. M., Nyborg, J., Thirup, S. & Clark, B. F. C. *EMBO J.* **4**, 2385–2388 (1985).
- Jurnak, F. *Science* **230**, 32–36 (1985).
- McCormick, F. *et al. Science* **230**, 78–82 (1985).
- Clark, R., Wong, G., Arnheim, N., Nitecki, D. & McCormick, F. *Proc. natn. Acad. Sci. U.S.A.* **82**, 5280–5284 (1985).
- Halliday, K. R. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **9**, 435–448 (1983).
- Feuerstein, J., Kalbitzer, H. R., John, J., Goody, R. S. & Wittinghofer, A. *Eur. J. Biochem.* **162**, 49–55 (1987).
- Dreusick, D. & Schulz, G. E. *FEBS Lett.* **208**, 301–304 (1986).
- Reinstein, J., Brune, M. & Wittinghofer, A. *Biochemistry* **27**, 4712–4720 (1988).
- Feig, L. A., Pan, B.-T., Roberts, T. M. & Cooper, G. M. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4607–4611 (1986).
- Wittinghofer, A., Warren, W. F. & Leberman, R. *FEBS Lett.* **75**, 241–243 (1977).
- Lancelotti, G., Mayer, R. & Hélène, C. *J. Am. Chem. Soc.* **101**, 1569–1576 (1979).
- Der, C. D., Pan, B.-T. & Cooper, G. M. *Molec. cell Biol.* **6**, 3291–3294 (1986).
- Clanton, D. J., Hattori, S. & Shih, T. Y. *Proc. natn. Acad. Sci. U.S.A.* **83**, 5076–5080 (1986).
- Sigal, I. S. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 952–956 (1986).
- Der, C. D., Weissmann, B. & MacDonald, M. J. *Oncogene* **3**, 105–112 (1988).

- Hwang, Y. W. & Miller, D. L. *J. biol. Chem.* **262**, 13081–13085 (1987).
- John, J., Frech, M., Feuerstein, J., Goody, R. S. & Wittinghofer, A. in *Guanine-Nucleotide-Binding Proteins* (Plenum, New York, in the press).
- Miller, R. T., Masters, S. B., Sullivan, K. A., Beiderman, B. & Bourne, H. R. *Nature* **334**, 712–715 (1988).
- Kalbitzer, H. R., Goody, R. S. & Wittinghofer, A. *Eur. J. Biochem.* **141**, 591–597 (1984).
- Sigal, I. S., Gibbs, J. B., D'Alonzo, J. S. & Scolnick, E. M. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4725–4729 (1986).
- Cales, C., Hancock, J. F. & Hall, A. *Nature* **332**, 548–551 (1988).
- Adari, H., Lowy, D. R., Willumsen, B. M., Der, C. J. & McCormick, F. *Science* **240**, 518–521 (1988).
- Sigal, I. S. *et al. Cold Spring Harbor Symp. quant. Biol.* **53**, 863–869 (1988).
- Pingoud, A., Block, W., Wittinghofer, A., Wolf, H. & Fischer, E. *J. biol. Chem.* **257**, 11261–11267 (1982).
- Furth, M. E., Davis, L. J., Fleurdelys, E. M. & Scolnick, E. M. *J. Virol.* **43**, 294–304 (1982).
- Lacal, J. C. & Aaronson, S. A. *Molec. cell Biol.* **6**, 1002–1009 (1986).
- Der, C. J., Finkel, T. & Cooper, G. M. *Cell* **44**, 167–176 (1986).
- Feig, L. A. & Cooper, G. M. *Molec. cell Biol.* **8**, 2472–2478 (1988).
- Feuerstein, J., Goody, R. S. & Webb, M. R. *J. biol. Chem.* **264**, 6188–6190 (1989).
- Schlichting, I. *et al. Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Durbin, R. M. *et al. Science* **232**, 1127–1132 (1986).
- Kabsch, W. *J. appl. Crystallogr.* **21**, 916–924 (1988).
- Kabsch, W. *J. appl. Crystallogr.* **21**, 67–71 (1988).
- Jones, T. A. *J. appl. Crystallogr.* **11**, 268–272 (1978).
- Hendrickson, W. A. & Konert, J. H. in *Computing in Crystallography* (eds Diamond, R., Ramaseshan, S. & Venkatesan, K.) 13.01–13.23 (Indian Academy of Science, International Union of Crystallography, Bangalore, 1980).
- Read, R. J. *Acta crystallogr.* **A42**, 140–149 (1986).
- Brünger, A. T., Kuriyan, J. & Karplus, M. *Science* **235**, 458–460 (1987).
- Kabsch, W. & Sander, C. *Biopolymers* **22**, 2577–2637 (1983).
- Priestle, J. P. *J. appl. Crystallogr.* **21**, 572–576 (1988).

ACKNOWLEDGEMENTS. We thank A. Scherer for growing crystals, T. Gamble and W. Gebhard for assistance with computer programs, G. A. Petsko for help with the refinement routines, A. T. Jones for introducing one of us to FRODO, G. Eulefeld for artwork, R. S. Goody for supplying the analogues and many ideas and all our colleagues for discussion.

Luminosity segregation as a constraint on the theory of biased galaxy formation

David Valls-Gabaud^{*§}, Jean-Michel Alimi^{†‡}
& Alain Blanchard[†]

^{*} Institut d'Astrophysique de Paris, 98 bis Boulevard Arago,
F-75014 Paris, France

[†] D.A.E.C., Observatoire de Meudon, F-92195 Meudon Cédex, France

[‡] Centre de Physique Théorique, Ecole Polytechnique,
F-91128 Palaiseau Cédex, France

THE theory of biased galaxy formation with cold dark matter (CDM) is presently the most successful attempt to reproduce the distribution of galaxies in the Universe¹⁻⁶, permitting a reconciliation of the observed low value of the cosmological density parameter $\Omega_0 \leq 0.2$ with the value of unity predicted by inflation. Specific tests of the bias hypothesis, namely that galaxies are more clustered than the mass^{1,2,4}, however, have not yet led to definitive conclusions. Here we show that a systematic luminosity segregation effect in the clustering of galaxies is expected and provides an unambiguous test of the biased galaxy formation theory. We examine in detail how the clustering of galaxies correlates with their luminosity, using different biased models and numerical simulations⁵⁻⁷. We find that a strong effect is expected at luminosities larger than L_* , the characteristic luminosity of galaxies and that the amplitude of the predicted effect at these luminosities agrees with observations⁸. At luminosities fainter than L_* , however, strong limits have been put on this segregation effect⁹. These limits are incompatible with any biased CDM theory in which the bias parameter b_b for bright galaxies is greater than 1.5 on large scales.

Many properties of the observed galaxy distribution are well reproduced by the CDM theory of galaxy formation¹⁰. The predicted spectrum of the density fluctuations in the early Universe seems to be precisely that required to explain both the small-scale and large-scale structure of this distribution. To reconcile the value of the cosmic density parameter $\Omega_0 = 1$ predicted by inflation with observations, however, the galaxies must be more clustered than the total mass. This bias may be the result of physical processes that took place at the time of galaxy formation^{2,4}, and is also a natural feature that arises in hierarchical theories^{6,11,12}. According to this bias hypothesis, the correlation function of bright galaxies ξ_{gg} is amplified with respect to the correlation function of the density field $\xi_{gg}(r) = b^2 \xi_{pp}(r)$ where the bias parameter b may depend on the distance r . For bright galaxies (that is, galaxies typically brighter than the absolute blue magnitude $M = -18.5 + 5 \log h$, where h is the Hubble constant in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$) this bias b_b depends on the specific model^{1,13} but must lie in the range 1.7–2.5 to make the dynamical measures of the density parameter compatible with the value of unity predicted by inflationary theories. Dwarf galaxies are expected to be more reliable tracers of the mass distribution³ than are bright galaxies, and their bias parameter b_d should therefore be much smaller than b_b and closer to unity. Numerical simulations in the CDM scenario predict that the strength of clustering correlates with the circular velocity of haloes⁶, and, therefore, with luminosity through the Tully–Fisher and Faber–Jackson relations⁷. Slowly rotating haloes ($v_c \approx 75 \text{ km s}^{-1}$) characteristic of dwarf galaxies, correspond to a bias parameter of about unity. Therefore, a luminosity segregation effect in the clustering of galaxies is expected in this biased CDM theory, as in any biased theory.

Until recently, the observational evidence for such an effect was ambiguous. Early qualitative analyses rejected the possibil-

ity of luminosity segregation^{14,15} and recent quantitative studies do not provide any clear indication^{16,17}. The correlation of surface brightness with the differences in clustering has been attributed to biasing¹⁸, but the effect remains controversial¹⁹. Actually, a luminosity segregation effect is observed on short scales²⁰⁻²², but this may be the result of dynamical friction, gravitational relaxation and other non-linear effects in addition to biasing. Quantitative results on scales $> 3h^{-1} \text{ Mpc}$, where the density field is still linear, have recently been obtained^{8,9} and can be compared directly, as below, with the theoretical predictions that result from analytical models and numerical simulations in the CDM scenario.

In biased CDM theories, bright galaxies correspond to the highest peaks of the initial density field, independent of the fact that the bias is 'natural'⁶ or a result of the physics of galaxy formation². As the density fluctuations generated by inflation are expected to be gaussian, the gaussian random field theory^{13,23} may be used to describe the distribution of galaxies on scales where dynamical evolution is not dominant. Galaxies are identified with the peaks of the density field of amplitude $\nu_{th} \sigma_0$ above the r.m.s. level σ_0 where ν_{th} is the chosen threshold. Numerical simulations have shown that this is a good approximation^{24,25} and that the associated peak–peak correlation function agrees with the observed correlation function of galaxies^{1,6}. On linear scales, the bias parameter is independent of distance and is given by $b = 1 + \langle \tilde{\nu} \rangle / \sigma_0(R_s)$, where R_s is the smoothing scale associated with galaxies. Exact expressions for calculating the mean effective threshold $\langle \tilde{\nu} \rangle$ are given in ref. 13. To determine peak–luminosity relations, the sharp-threshold criterion may be too extreme, however, as the luminosity is probably not determined unequivocally by the amplitude of the peak. Nevertheless, a correlation between luminosity and the height of the peaks must remain—otherwise the galaxy distribution would no longer be biased. Thus, we introduce a selection function that gives the probability that a peak of a given amplitude $\nu \sigma_0$ actually forms a galaxy of a given luminosity, independent of the formation process. Using the selection function $t(\nu) = (\nu/\nu_{th})^q / (1 + (\nu/\nu_{th})^q)$ parameterized by the factor q (ref. 13), the sharp criterion is recovered as the parameter $q \rightarrow \infty$; finite values of q allow a dispersion in the relation between the amplitude of the peaks and the luminosity of galaxies. Adopting a smoothing scale R_s^* associated with bright galaxies, the normalization threshold ν_{th}^* for these galaxies is obtained by requiring that the number n_{pk} of peaks above this threshold equals the observed number n_b of bright galaxies $n_{pk}(\nu > \nu_{th}^*, R_s^*) = n_b$. The number of peaks n_{pk} is estimated for the CDM power spectrum¹³, and n_b is normalized to $0.01 h^3 \text{ Mpc}^{-3}$ (ref. 26). A constant smoothing scale R_s is unphysical as the number of galaxies apparently diverges at the faint end of the luminosity function, but the total number of peaks remains finite. A simple power-law dependence $R_s \approx L^a$ is therefore assumed. If the mass associated with the peak scales as R_s^3 (ref. 23), a constant mass-to-light ratio favours $a = \frac{1}{3}$, whereas a maximum value of 0.5 is allowed by observations¹⁰. Following the Press and Schechter formalism^{27,28}, we determine the threshold ν_{th} at each luminosity by requiring that the total mass in the selected peaks matches the total mass in the galaxies

$$R_s^3 n_{pk}(\nu > \nu_{th}, R_s) \propto \int_{L(R_s)}^{+\infty} L^{3a} \Phi(L) dL \quad (1)$$

where $\Phi(L)$ is the luminosity function. This prescription allows the estimation of the dependence of the bias parameter on luminosity.

Figure 1 shows a comparison of predictions from the biased models described above with the observed strong luminosity segregation effect found⁸ at luminosities brighter than L_* (corresponding to $M_* \approx -19.4 - 5 \log h$). The expected segregation derived from numerical simulations⁶ is also shown, where the simulated circular velocity of haloes has been converted into

[§] Present address: Institute of Astronomy, Madingley Road, Cambridge CB3 0HA, UK

luminosity through the Faber-Jackson and Tully-Fisher relations²², taking into account the proper number density of galaxies of a given velocity or luminosity. Given the uncertainties in the measures of bias, theoretical predictions with $b_b \approx 2$ agree well with the observed trend illustrated in Fig. 1, whereas models with low values of b_b and q (corresponding to large peak-luminosity dispersions) fail to reproduce the strong segregation found at high luminosities. The effect remains even when a gaussian dispersion is introduced into the velocity-luminosity relation from numerical simulations. It can also be observed that both models and simulations predict a non-negligible effect at luminosities fainter than L_* independent of the choice of the parameters. As shown below, this may constitute a serious problem for biased CDM scenarios as no significant luminosity segregation has been observed at luminosities fainter than L_* (refs 8, 9).

Because of the small amplitude of the predicted effect at low luminosities, it is preferable to use the ratio α of the cross-correlation functions, as this measure of the segregation effect is much less sensitive to sampling fluctuations and to normalization uncertainties in the measure of the correlation function^{9,29}. The cross-correlation of galaxies brighter than L_1 with galaxies brighter than L_2 is $\xi_{L_1, L_2}(r) = b(L_1)b(L_2)\xi_{p,p}(r)$. The relevant quantity to be compared with observations is $\alpha(L_2, L_1) = b(L_1)/b(L_2)$ where the luminosity L_1 is one magnitude fainter than L_2 (ref. 9). A large set of models was calculated, exploring the full parameter space ($a = 0-0.5$; $q = 4-\infty$; $R_s = 0.05-0.5h^{-1}$ Mpc). The adoption of different luminosity functions for present galaxies makes only a small difference.

Whereas the behaviour at bright luminosities depends strongly on the selection function, the trend of α is remarkably stable at fainter luminosities and depends essentially on b_b , the parameter on which reliable conclusions can be based. This arises from the fact that a correlation between high peaks and bright galaxies always exists in any biased theory. As illustrated in Fig. 2, the only model that is not strongly excluded by the data has $b_b \approx 1.5$. This extreme model predicts $b^2(-19)/b^2(-15) \approx 1.27$, which is marginally consistent with the observed limits of $b^2(-19)/b^2(-15) \approx 0.993 \pm 0.145$, derived from volume-limited

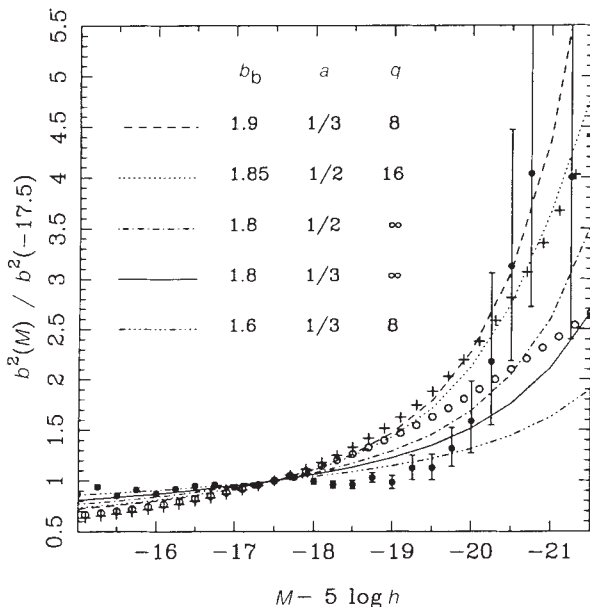


FIG. 1 Comparison of the observed variation (filled circles) of the bias parameter⁸ at $4h^{-1}$ Mpc (normalized to its value at magnitude $-17.5 - 5 \log h$) with theoretical predictions. Numerical simulations⁶ are represented by open circles (one-magnitude gaussian dispersion in the circular velocity-luminosity relation) and crosses (no dispersion). The lines correspond to different biased models as indicated in the key.

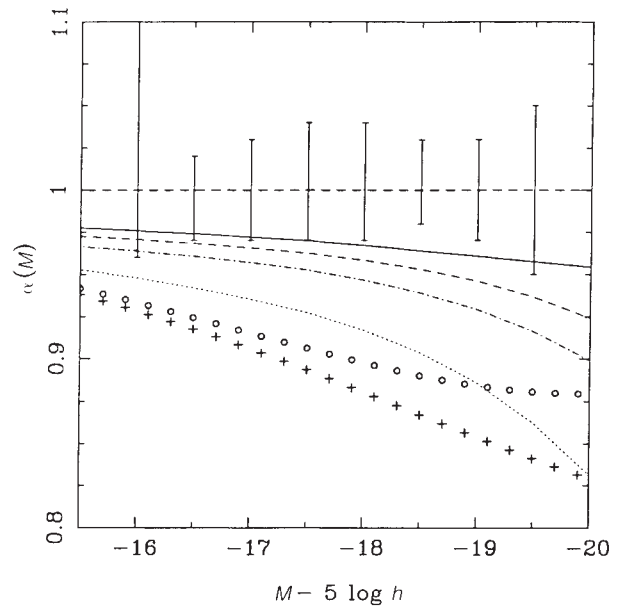


FIG. 2 Comparison of the observed variation of the alpha ratio⁹ with biased models (full: $b_b = 1.5$, $a = \frac{1}{3}$, $q = 8$; dashed: $b_b = 1.7$, $a = \frac{1}{3}$, $q \rightarrow \infty$; dash-dotted: $b_b = 1.8$, $a = \frac{1}{3}$, $q \rightarrow \infty$; dotted: $b_b = 1.9$, $a = \frac{1}{3}$, $q = 8$) and numerical simulations⁶ (as in Fig. 1). The value $\alpha = 1$ (thick dashes) is the most likely to be compatible with observations.

subsamples in the survey²⁶, and extensively discussed in ref. 9. Figure 2 shows that the absence of segregation ($\alpha = 1$) is much more likely than any biased model. It is possible to obtain extreme models which are compatible with the data at a significant level, but their bias parameter b_b is always < 1.5 . For instance, a smoothing scale as small as $R_s \leq 0.05h^{-1}$ Mpc may be used for bright galaxies, but then the baryonic fraction enclosed in these fluctuations would be very small ($\leq 2.5 \times 10^{-4} \Omega_0$) and would require an important secondary infall, which seems unlikely.

The agreement of biased CDM models of galaxy formation with a large set of observations is impressive, despite some discrepancies with recent observations of large-scale phenomena. In particular, the correlation length of clusters³⁰ and large-scale streaming motions³¹ may well be in conflict with this theory, but the interpretation of these observations is still uncertain³². Even if these observations are interesting tests of the density spectrum shape, they do not directly test the bias hypothesis. The dependence of the galaxy clustering on luminosity seems to be inherent to any theory in which galaxies are not distributed as the mass. Therefore, the luminosity segregation effect provides a critical test that does not depend on the details of the physical processes that lead to galaxy formation, but follows directly from the hypothesis that galaxies are more clustered than the underlying mass. We have shown that biased theories in the CDM scenario with b_b larger than 1.5 are in clear conflict with observations, but the resolution of the cosmic density parameter requires $b_b \approx 2.5$ (ref. 1). It is nonetheless desirable to extend the observational analysis to larger samples before drawing any definitive conclusions, as the presently available catalogues offer only a small volume for the analysis of the effect. Also, the hypothesis that the clustering in bright galaxies does not match the mass distribution is still possible on short scales because, in the CDM theory, the fraction of total mass in visible baryons is small. Nevertheless, we have found limits on the luminosity segregation effect in the clustering of galaxies on large scales that are incompatible with the present versions

of the theory of biased galaxy formation with cold dark matter. □

Received 27 February; accepted 18 August 1989.

1. Davis, M., Efstathiou, G., Frenk, C. S. & White, S. D. M. *Astrophys. J.* **292**, 371–394 (1985).
2. Rees, M. J. *Mon. Not. R. astr. Soc.* **213**, 75p–81p (1985).
3. Dekel, A. & Silk, J. *Astrophys. J.* **303**, 39–55 (1986).
4. Dekel, A. & Rees, M. J. *Nature* **326**, 455–462 (1987).
5. Frenk, C. S., White, S. D. M., Efstathiou, G. & Davis, M. *Nature* **317**, 595–597 (1985).
6. White, S. D. M., Davis, M., Efstathiou, G. & Frenk, C. S. *Nature* **330**, 451–453 (1987).
7. Frenk, C. S., White, S. D. M., Davis, M. & Efstathiou, G. *Astrophys. J.* **327**, 507–525 (1988).
8. Hamilton, A. J. S. *Astrophys. J.* **331**, L59–L62 (1988).
9. Alimi, J.-M., Vallis-Gabaud, D. & Blanchard, A. *Astr. Astrophys.* **206**, L11–L14 (1988).
10. Blumenthal, G. R., Faber, S. M., Primack, J. R. & Rees, M. J. *Nature* **311**, 517–525 (1984).
11. Schaeffer, R. *Astr. Astrophys.* **180**, L5–L8 (1987).
12. West, M. J. & Richstone, D. O. *Astrophys. J.* **335**, 532–541 (1988).
13. Bardeen, J. M., Bond, J. R., Kaiser, N. & Szalay, A. S. *Astrophys. J.* **304**, 15–61 (1986).
14. Davis, M., Huchra, J., Latham, D. W. & Tonry, J. *Astrophys. J.* **253**, 423–445 (1982).
15. Einasto, J., Klypin, A. & Saar, E. *Mon. Not. R. astr. Soc.* **219**, 457–478 (1986).
16. Philipps, S. & Shanks, T. *Mon. Not. R. astr. Soc.* **229**, 621–626 (1987).
17. Einasto, M. *Mon. Not. R. astr. Soc.* **234**, 37–50 (1988).
18. Davis, M. & Djorgovski, S. *Astrophys. J.* **299**, 15–20 (1985).
19. Bothun, G. D., Beers, T. G., Mould, J. R. & Huchra, J. P. *Astrophys. J.* **308**, 510–529 (1986).
20. Tully, B. *Astr. J.* **96**, 73–80 (1988).
21. Davis, M., Melnick, A., Strauss, M. A., Da Costa, L. N. & Yahil, A. *Astrophys. J.* **333**, L9–L12 (1988).
22. Davis, M., Tully, B. & White, S. D. M. *Astrophys. J.* **333**, L45–L49 (1988).
23. Peacock, J. A. & Heavens, A. F. *Mon. Not. R. astr. Soc.* **217**, 805–820 (1985).
24. White, S. D. M. in *The Early Universe* (eds Urru, W. G. & Semanoff, G. W.) 239–260 (Reidel, Dordrecht, 1988).
25. Carlberg, R. G. & Couchman, H. M. P. *Astrophys. J.* **340**, 47–68 (1989).
26. Davis, M. & Peebles, P. J. E. *Astrophys. J.* **267**, 465–482 (1983).
27. Press, W. H. & Schechter, P. *Astrophys. J.* **187**, 425–438 (1974).
28. Schaeffer, R. & Silk, J. *Astrophys. J.* **292**, 319–329 (1985).
29. Blanchard, A. & Alimi, J.-M. *Astr. Astrophys.* **203**, L1–L4 (1988).
30. Bahcall, N. A. & Soneira, R. M. *Astrophys. J.* **270**, 20–38 (1983).
31. Lynden-Bell, D. et al. *Astrophys. J.* **326**, 50–62 (1988).
32. Sutherland, W. *Mon. Not. R. astr. Soc.* **234**, 159–172 (1988).

ACKNOWLEDGEMENTS. We are grateful to A. Hamilton for a copy of his measures of the relative bias and J. Silk for comments.

Molecular hydrogen emission from the bright quasar 3C273

Kimiaki Kawara*, Minoru Nishida†|| & Brooke Gregory‡§

* National Astronomical Observatory, Mitaka, Tokyo 181, Japan

† Department of Physics, Kyoto University, Kyoto 606, Japan

‡ Cerro Tololo Inter-American Observatory,

National Optical Astronomy Observatories, Casilla 603, La Serena, Chile

§ Royal Observatory, Blackford Hill, Edinburgh EH9 3HJ, UK

THE discovery of broad emission lines in polarized light from the type 2 Seyfert galaxy NGC1068 led Antonucci and Miller¹ to postulate the existence of a type 1 Seyfert nucleus shrouded from our direct view by a torus of molecular gas. Theoretical development² of this idea included the suggestion that low-angular-momentum clouds in the torus are captured by the central source, fuelling the observed activity. The difficulty in applying this model to all active galactic nuclei (AGNs) is the lack of convincing evidence for molecular gas in 'bare' nucleus objects such as the quasar 3C273, which exhibits a simple power-law continuum and no excess of thermal dust emission. Here we present observations, made during the course of a survey for rotation and vibration lines of H₂ emission from type 1 Seyferts and quasars, of molecular hydrogen emission from 3C273. This is the first time such emission has been seen from a radio-loud quasar.

The H₂ line used was the $v=1-0$ S(3) transition (rest wavelength 1.957559 μm (ref. 3)), instead of the $v=1-0$ S(1) line which has been well studied and is one of the brightest H₂ emission lines. We chose this line because the frequency of redshifted $v=1-0$ S(1) line (2.122 μm) falls outside the trans-

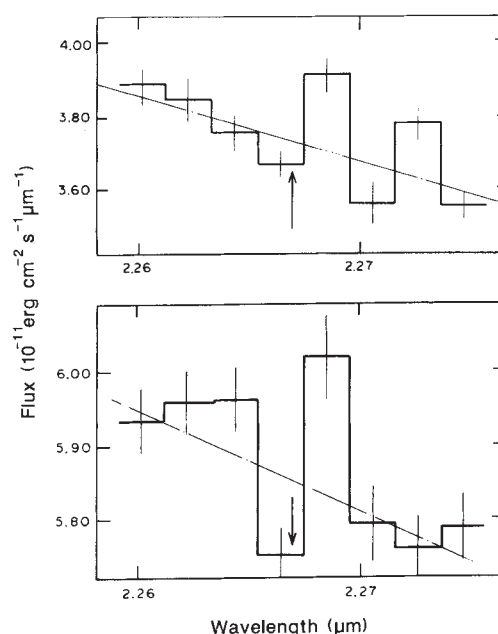


FIG. 1 Spectra taken in December 1986 and February 1988 (top and bottom respectively) near the position of the $v=1-0$ S(3) lines. Uncertainties correspond to 1σ statistical noise. The data are sampled with one point per instrumental resolution element. The straight line is the continuum level used to calculate the line flux. The arrow shows the predicted line position based on optical data.

mission window of the Earth's atmosphere.

We observed the line on two occasions, 1986 December 27.36 and 1988 February 2.33 (UT) using the Cerro Tololo Inter-American Observatory 4-m telescope equipped with the infrared spectrometer⁴ (IRS). The broad-band K-magnitudes were obtained simultaneously, using the monitor channel of the IRS. A 3.4×6.8 inch slit was used (where the first dimension was parallel to the dispersion axis). The frequency resolution of the IRS corresponds to a velocity resolution of 290 km s^{-1} per detector. We used a xenon or an argon lamp to calibrate the wavelength scale before each integration and achieved an accuracy of 10% of the resolution (30 km s^{-1}). The spectroscopic data is sampled with one point per instrumental resolution element. The Am-type star BS4167 (ref. 5) was used to calibrate the flux scale and correct for atmospheric extinction. The spectroscopic data were obtained on nights when the photometric condition was good and the accuracy of the flux is 5% or better. There are no stellar absorption features or serious telluric absorptions in the standard spectrum.

Figure 1 shows the H₂ spectra; the arrow shows the line position predicted from the optical data⁶. The straight line in the figure is the continuum level used to obtain the line flux given in Table 1. The continuum line is a linear fit to assumed continuum points on either side of the emission line, weighted by the inverse squares of the uncertainties.

During the December 1986 run, 3C273 was in a quiescent state and its K-magnitude was 9.74 or 80 mJy whereas on 2 February 1988, its K-magnitude was 9.50 or 100 mJy. At the end of January 1988, the J-band flux was 10% higher than that of the quiescent state⁷. It continued to increase gradually in February, reaching maximum brightness in mid-March⁷. Our record of the increase in the K-flux indicates that the luminosity from 1.2 μm to 2.2 μm started to increase simultaneously during this event. The continuum near the H₂ line brightened by 0.49 mag (see Fig. 1), 0.25 mag more than the integrated K-flux.

The $v=1-0$ S(3) line was detected in each run. Because of the low signal-to-noise ratios we are not certain that the line fluxes in both runs remained constant. It is probable, however, that the line fluxes did not change during the flare, because the

|| To whom correspondence should be addressed.

molecular clouds that are the source of the H_2 emission are probably located at a distance >1 pc from the central non-thermal source². The spectra of the two runs (see Fig. 2) were combined by reducing the continuum level in February 1989 spectrum by 0.49 mag and assuming the line flux to be constant. The resultant spectrum has a $v=1-0$ S(3) line flux of $(4.14 \pm 0.58) \times 10^{-15}$ erg cm⁻² s⁻¹. The continuum of Fig. 2 refers to that in the quiescent state. Scaling the entire February 1988 spectrum by 0.49 mag results in a low line flux of $(3.20 \pm 0.58) \times 10^{-15}$ erg cm⁻² s⁻¹. The predicted line position is offset by ~ 200 km s⁻¹ from the centre of the line pixel. This may be the result of an uncertainty in the determination of the velocity shift of the optical lines or to differences in the velocity between optical and H_2 emission lines. We note that the slope of the continuum is steep. This may be the result of the broad wing of the Br δ line (1.94456- μ m rest wavelength) which is only 2,000 km s⁻¹ to the blue of the $v=1-0$ S(3) line.

We assume the ratio of $v=1-0$ S(3) to $v=1-0$ S(1) fluxes to be unity in order to compare our results with other observations. Within the uncertainty of the line flux, this assumption is consistent with the predictions from shock excitation^{8,9} or X-ray heating¹⁰ models, both of which are plausible mechanisms for H_2 excitation in AGNs^{4,11}. This ratio is 0.77 (ref. 12) in the merging galaxy NGC6240. The mass of hot infrared-emitting H_2 molecules can be roughly estimated by assuming a temperature of 2,000 K for the H_2 molecular gas¹³. It is $8 \times 10^4 M_\odot$ at a distance of 630 Mpc assuming a Hubble constant $H_0 = 75$ km s⁻¹ Mpc⁻¹ and a deceleration parameter $q_0 = 0$.

Molecular hydrogen emission, direct evidence for the presence of a considerable mass of hot molecular gas in 3C273, has not been previously reported. Here we discuss possible relationships between H_2 emission and AGN activity in 3C273. We have found⁴ that the molecular hydrogen emission is generally stronger in dusty AGNs such as type 2 Seyferts than in H II-region galaxies when normalized by the IRAS far-infrared flux¹⁴. This separation is clear in the subsample observed with a small aperture the diameter of which is $\leq 6\%$ of the isophotal galaxy diameter defined by a surface brightness of 25 mag arcsec⁻²; with increasing aperture the differences between AGN and H II region galaxies disappear. The ratio of the H_2 line to the far-infrared flux in 3C273 is 3.8×10^{-5} , which is about the maximum for dusty AGN and H II region galaxies. For dusty AGNs, the far-infrared flux is a measure of the quantity of warm dust or energy input to molecular clouds, and thus the ratio of the H_2 line to far-infrared flux is a measure of the efficiency of H_2 excitation. The observed far-infrared flux of 3C273 should be corrected because 3C273 seems to be completely dominated by non-thermal emission in the far-infrared. Extrapolating the power law from the data between millimetre and submillimetre wavelengths⁶, we estimated the upper limit of the thermal contribution to the observed far-infrared flux to be 15%. If we correct

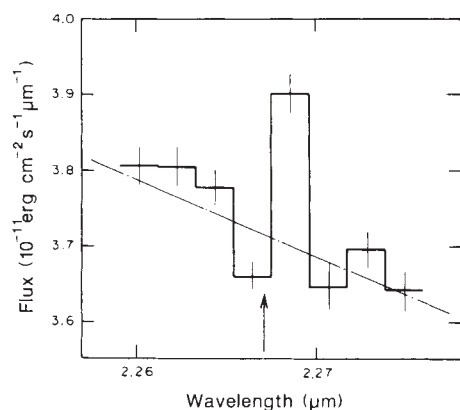


FIG. 2 As for Fig. 1, but for the combined spectrum of the $v=1-0$ S(3) line (see text).

TABLE 1 Observations of 3C273

Date (UT)	K-magnitude	$v=1-0$ S(3) (10^{-15} erg cm ⁻² s ⁻¹)
1986 December 27.36	9.74	4.6 ± 1.3
1988 February 2.33	9.50	3.9 ± 1.4
Average (from combined spectrum)		4.1 ± 0.6

for this effect, the efficiency for H_2 excitation or the ratio of H_2 to warm-dust luminosities becomes $\geq 2.5 \times 10^{-4}$, which is the largest efficiency ever observed. Hence, it is quite likely that H_2 emission in 3C273 is closely related to the activity of the central source.

If molecular clouds exist near the central source as suggested by the molecular torus models², the clouds directly heated by radiation from the central source would be hot enough to radiate H_2 emission and thermal continuum peaked in the near infrared. The prominent bump in the continuum at 3.5 μ m may be relevant here because it is believed to be the result of either free-free or thermal dust emission. The blackbody radiation from hot dust fits¹⁵ the 3.5- μ m bump better than free-free emission from a region of density 10^{11} cm⁻³ (ref. 16). Barvainis¹⁷ developed models to account for the 3.5- μ m bump of 3C273 in terms of thermal radiation from dust heated by an optical-ultraviolet continuum. In his model, grains are heated to $T = 1,500$ K at a radius of ~ 1 pc from the central source. Inside this radius, grains are evaporated by intense ultraviolet radiation. The mass of the grains in 3C273 calculated from these models is $7 \times 10^2 M_\odot$ at $T = 320-1,500$ K. If the dust-to-gas ratio is similar to the Milky Way and the hot molecular gas and the hot dust are mixed, our results are consistent with his estimates. It is uncertain that the H_2 -emission region in 3C273 is located at such a close distance from the central source, however, because the slit used in our observations corresponds to a physical size of 10×20 kpc. We note that observations¹⁸ of 18 type 1 Seyferts and quasars suggest that the spatial extent of H_2 -emission regions is small or unresolved, which is in contrast to type 2 Seyferts and H II region galaxies, the H_2 -emission regions of which are considerably extended¹². This is only circumstantial evidence for the relationship between H_2 emission and the dust-emission models, and thus it is important to observe the H_2 emission of nearby type 1 Seyferts that have no thermal emission in the far-infrared by using a small aperture. Through the idea² that low-angular-momentum clouds are captured by (and therefore feed) the central source, the H_2 emission from this class of objects may help us to understand the fuelling of AGNs¹⁹. □

Received 13 April; accepted 10 August 1989.

- Antonucci, R. R. J. & Miller, J. S. *Astrophys. J.* **297**, 621-632 (1985).
- Krolik, J. H. & Begelman, M. C. *Astrophys. J.* **329**, 702-711 (1988).
- Bragg, S. L., Brault, J. W. & Smith, W. H. *Astrophys. J.* **263**, 999-1004 (1982).
- Kawara, K., Nishida, M. & Gregory, B. *Astrophys. J.* **321**, L35-L40 (1987).
- Elias, J. H., Frogel, J. A., Matthews, K. & Neugebauer, G. *Astr. J.* **87**, 1029-1034 (1982).
- Sanders, D. B. et al. *Astrophys. J.* **328**, L35-L40 (1988).
- Courvoisier, T. J.-L. et al. *Nature* **335**, 330-333 (1988).
- Kwan, J. *Astrophys. J.* **216**, 713-723 (1977).
- Hollenbach, D. J. & Shull, J. M. *Astrophys. J.* **216**, 419-426 (1977).
- Lepp, S. & McCray, R. *Astrophys. J.* **269**, 560-567 (1983).
- Kawara, K., Nishida, M. & Gregory, B. *Astrophys. J.* **342**, L55-L58 (1989).
- Joseph, R. D. et al. in *Star Formation in Galaxies*, 421-433 (National Space and Aeronautics Administration, Washington, 1987).
- Scoville, N. Z., Hall, D. N. B., Kleinmann, S. G. & Ridgway, S. T. *Astrophys. J.* **253**, 136-148 (1982).
- Joint Infrared Astronomical Satellite Science Working Group *IRAS Point-Source Catalog* (U.S. Government Printing Office, Washington DC, 1985).
- Carleton, N. P. et al. *Astrophys. J.* **318**, 595-611 (1987).
- Puetter, R. C. & Hubbard, E. N. *Astrophys. J.* **295**, 394-401 (1985).
- Barvainis, R. *Astrophys. J.* **320**, 537-544 (1987).
- Kawara, K., Nishida, M. & Gregory, B. *Astrophys. J.* (submitted).
- Begelman, M. C., Blandford, R. D. & Rees, M. J. *Rev. mod. Phys.* **56**, 255-351 (1984).

ACKNOWLEDGEMENTS. We thank the staff of CTIO for their assistance and Y. Taniguchi for informing us of the flare in 3C273. K.K. and M.N. were supported by grants under the Monbusho International Scientific Research Program (Japan). K.K. and M.N. are Visiting Astronomers at CTIO. CTIO is operated by The Association of Universities for Research in Astronomy, Inc. under contract from the NSF.

VLF emission bursts in the terrestrial and venusian nightside troughs

Kaichi Maeda & Joseph M. Grebowsky

NASA/Goddard Space Flight Center, Greenbelt, Maryland 20771, USA

THE existence of very-low-frequency (~ 100 Hz) electric-field bursts in the nightside Venus ionosphere has been commonly assumed to be evidence for lightning, by analogy to the Earth where some of the whistler-mode radiations above the ionosphere originate from lightning¹⁻³. Non-lightning sources for Venus whistler-mode waves have been ignored. VLF emission bursts not associated with lightning are frequently observed over the terrestrial polar ionosphere⁴⁻⁶. One type, saucers, are whistler-mode wave emissions that propagate upwards along magnetic-field lines^{5,6}. Here we show that the 100-Hz emission bursts in the venusian nightside ionosphere are remarkably similar to those of the terrestrial VLF saucer emissions in terms of their spatial and temporal characteristics and the properties of their magnetoplasma environment. We therefore propose that the generation mechanism for such VLF saucers^{6,7} should be considered as a viable alternative to lightning as a source of venusian ionospheric whistler-mode emissions.

The VLF emission data obtained from terrestrial polar-orbiting satellites show bursts of signal. Some of these signals are V-shaped in wide-band data with frequencies below the lower of the local electron gyrofrequency or plasma frequency, that is, the missions are whistler-mode waves. Two kinds of whistler modes—V-shaped saucers and V-shaped hiss—are observed⁴⁻⁶. The hiss in the polar region is related to intense electron precipitation and is Cerenkov radiation from auroral electrons. Hiss propagates downwards. The V-shaped saucer which propagates upward along magnetic-field lines, however, is not related directly to auroral-particle precipitation and is observed above or within nightside polar ionospheric troughs⁵. The saucers can be produced by the cyclotron instability of an anisotropic distribution of hot electrons interacting within the polar upper ionosphere⁷ or by suprathermal electrons streaming upward through the background plasma⁶.

Figure 1 shows the wide-band a.c. electric-field data of a V-shaped saucer event observed by the DE-1 satellite on 27 January 1984. The a.c. magnetic-field data are not presented but the V-shaped emissions in this wide-band data are known to be whistler-mode electromagnetic waves⁵. There are also several sharp slanted emission lines appearing around the main V-

FIG. 1 The wide-band a.c. electric-field data of V-shaped saucer with several V-type emission events on 27 January 1984 observed from the DE-1 satellite. Under the data are: time; h , satellite altitude; R , radial distance of the satellite normalized by the Earth's radius ($R_E = 6,371$ km); L , dipole-field parameter; Lat., invariant latitude; MLT, magnetic local time; f_g , electron gyrofrequency; f_p , electron plasma frequency; ratio f_p/f_g .

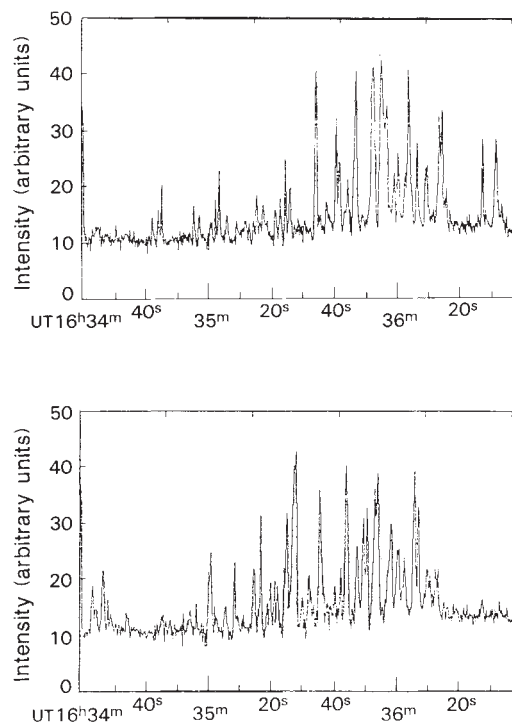
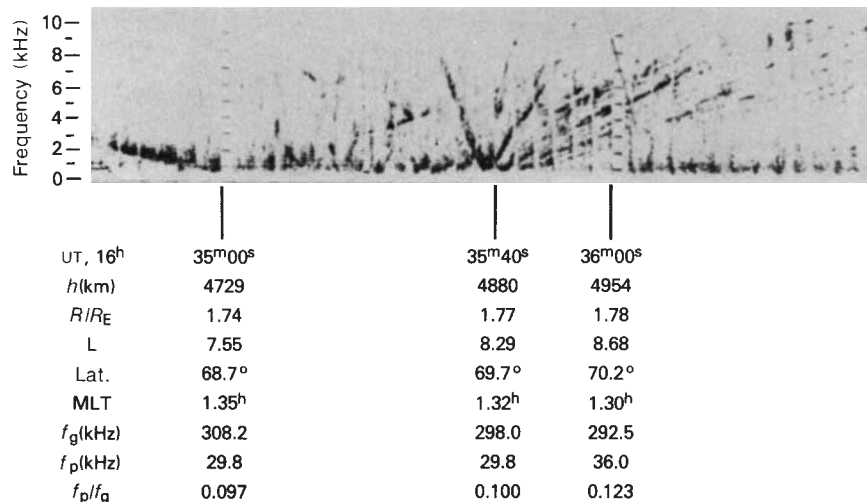


FIG. 2 The microdensitometer trace of the wide-band data (Fig. 1) at constant frequencies of 4 kHz (top) and 6 kHz (bottom) with 4% bandwidth.

shaped event, the apex of which appears at 16:35:40 UT. These emission bursts are one side of several V-shaped saucers, the apexes of which occur around 16:35:20 UT. The detection of only one side, or only part of one side, of these V-shaped saucers from a high-velocity satellite indicates the short durations of the generation of these emissions, that is, unsteady plasma conditions in the source region. These burst features are attributed to characteristics of the source electrons and of the irregularities in the plasma density distribution in the polar upper ionosphere. The irregularities in the plasma density are sustained predominantly only in the night-time ionosphere. The large aperture angle of these background features is a manifestation of the large distance of the sources of these emissions from the satellite^{6,7}. These events occur in the nightside polar ionospheric troughs, as indicated by the very small ratio of the plasma frequency f_p to the electron gyrofrequency f_g , that is, $f_p/f_g \ll 1$. Similar findings that indicate the appearance of VLF

saucers in the nightside polar ionospheric troughs were made by the OGO-6 experiments (K.M. and J.M.G., manuscript in preparation).

The 'bursty' nature of V-shaped saucers shown in Fig. 1 can be seen more clearly from Fig. 2 where the relative intensity variations of the emission within fixed frequency bands are presented; this is the type of electric-field measurements made from the Pioneer Venus Orbiter (PVO), presented later. This presentation is produced by scanning the wide-band data shown in Fig. 1 at frequencies of 6 kHz and 4 kHz (4% bandwidth) with a microdensitometer. Here, the slit width (pixel size) of the microdensitometer corresponds to the bandwidth of the VLF detector. These figures show the 'bursty' nature of the saucers. Some of the spiking in Fig. 2 is associated with the satellite-spin modulation of the wide-band data shown in Fig. 1, and can be easily distinguished by comparing upper and lower figures, that is, the natural bursts appear at different instants at 4 kHz and 6 kHz, but the 3-s modulation that results from the satellite's 10-r.p.m. spin occurs at the same instants in both figures.

An example of the VLF emission data from the PVO within the nightside venusian ionosphere is presented in Fig. 3. The Orbital Electric Field Detector (OEFD) measures intensities within four frequency bands that have a 30% bandwidth centred on 31, 5.4, 0.730 and 0.100 kHz. On the bottom, the total ion density N_T (ions cm^{-3}) measurements made simultaneously by the orbiter ion mass spectrometer (OIMS) are shown. This figure shows an association between the 100-Hz emissions occurring in the absence of higher-frequency emissions and a plasma trough—this is characteristic of the emission^{8,9}. It should be noted that the 100-Hz emission is the only possible whistler-

mode wave among the venusian ionospheric emissions observed² by the four-channel plasma-wave instrument (OEFD) aboard the PVO.

The characteristics of the 100-Hz band emissions are clearly different from the other three emissions of higher frequency (Fig. 3). The enhanced 100-Hz emission bursts are observed within the ion troughs which exist only in the nightside venusian ionosphere and within which the magnetic field is enhanced, projected toward the planet and is characterized by a gyro-frequency that is generally >100 Hz. Unlike the 100-Hz emissions, the emissions above 0.73 kHz are enhanced when the PVO approaches its periapsis, which is consistent with local CO_2 impact generation of ion acoustic waves¹⁰. The spike-like 100-Hz emissions that were classified as lightning bursts⁹ are shown by vertical lines under the 100-Hz emission.

For the terrestrial saucer emissions, the frequency can extend from the lower hybrid resonance frequency⁶ that is of the order of 100 Hz up to the electron gyrofrequency or plasma frequency (both of the order of several hundred kHz). This is the domain of the whistler-mode wave in the terrestrial polar ionosphere and magnetosphere. Because Venus does not possess an intrinsic magnetic-dipole field, the radial nightside static magnetic fields are the interplanetary magnetic fields (IMF) brought in by the solar wind. The corresponding electron gyrofrequencies that define the upper limit of venusian whistler-mode emissions are of the order of 100 Hz for these rather weak induced fields.

A comparison of the variation of the near monochromatic intensities of the terrestrial VLF saucers shown in Fig. 2 to the 100-Hz Venus emissions shown in Fig. 3 shows a striking similarity in the burst characteristic of these two emissions. Other close similarities in features and characteristics of these emissions exist and are listed in Table 1. The lack of confirmation of the association of particle precipitations with terrestrial saucers and PVO 100-Hz emissions can be ascribed to the limitation of measurements; the existence of short-lived narrow beams of electron precipitation during these emission events cannot be ruled out at present. There are theoretical implications of this situation and connections with other phenomena such as the electrostatic shocks and the polar plasma cavities outside of the auroral activities in the terrestrial polar magnetosphere¹⁰⁻¹².

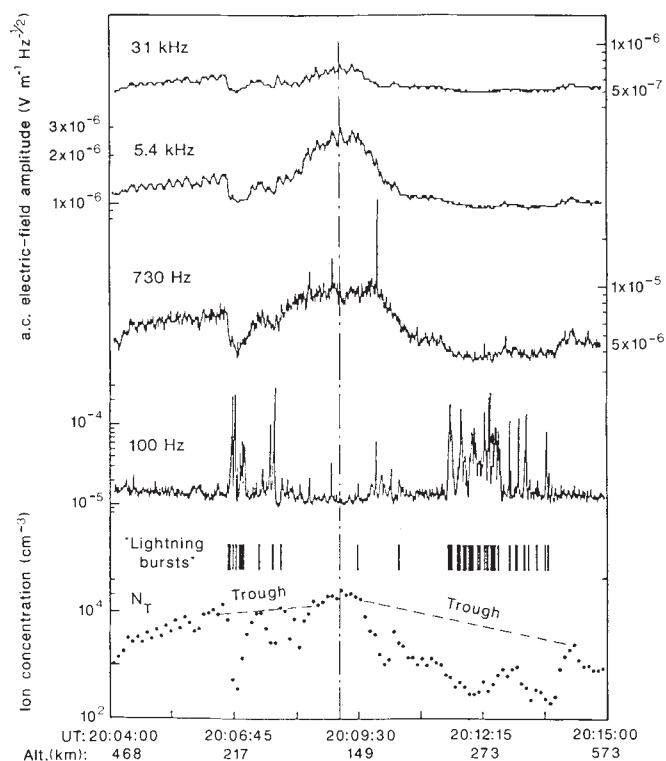


FIG. 3 Pioneer Venus orbiter (PVO) data of VLF emissions at 100 Hz, 730 Hz, 5.4 kHz and 31 kHz. The a.c. electric-field amplitude ($\text{V m}^{-1} \text{Hz}^{-1/2}$) appears on the left-hand axis and right-hand axis alternately. The total ion density (cm^{-3}) observed by the OIMS are also shown by dots for a period 20:04:00–20:15:00 UT on orbit 66. A dot-dashed vertical line in the centre indicates the passage of the periapsis. The altitude of PVO above the venusian surface is indicated under the time annotations. Emissions identified⁹ as lightning bursts based on OEFD data are shown by vertical lines under the 100-Hz intensity curve.

TABLE 1 Impulsive VLF properties

	Terrestrial VLF saucer	Venusian 100-Hz emission
Emission characteristics	$f < (f_g, f_p)_{\min}$; bursts; electromagnetic ⁴⁻⁶	$f < f_g$ (which is $< f_p$); bursts; electric-field component normal to static magnetic field ^{1,3}
Association with particle precipitation	Unconfirmed ^{5,6}	None evident
Propagation	Upward along magnetic field; right-handed polarization; whistler mode but weak magnetic component ⁵	Upward presumed; polarization unestablished but not inconsistent with whistler-mode propagation ²
Location	Nightside in ionospheric troughs where magnetic field is radial ⁵⁻⁷	Nightside in ionospheric holes where magnetic field is radial ^{1-3,8}
Duration	Individual emissions are a fraction of a second long; one event with an apex is <1 min (refs 5, 6)	Individual emissions are <0.5 s; event duration nominally <1 min (ref. 1)
Related phenomena	Electrostatic shock; narrow upward ion beams; suprathermal conical ion distributions ^{11,12}	Suprathermal ions around the ion holes ⁸

On the basis of the comparison of the known features of the terrestrial saucers and the venusian 100-Hz whistler bursts, it seems natural to conclude that the latter emissions in the venusian nightside ionospheric troughs can arise from mechanisms similar to production of VLF saucers in the terrestrial nightside polar ionospheric troughs. These mechanisms for the 100-Hz emission are just as plausible an explanation as that proposed by Scarf and Russell¹⁻³ who used analogies to terrestrial lightning events to associate the emissions with volcanic activities in high mountain clusters on the venusian surface. The validity of either of these mechanisms can be easily verified if frequency-time spectrograms (wide-band data) of VLF emissions in the nightside venusian ionosphere are obtained. Without such observations it will be very difficult to distinguish lightning disturbances from ionospheric generation of impulsive whistler bursts of energetic electrons. □

Received 5 May; accepted 10 August 1989.

1. Scarf, F. L., Taylor, W. W. L., Russell, C. T. & Brace, L. H. *J. geophys. Res.* **85**, 8158-8166 (1980).
2. Scarf, F. L. & Russell, C. T. *Geophys. Res. Lett.* **10**, 1192-1195 (1983).
3. Russell, C. T., von Dornum, M. & Scarf, F. L. *Nature* **331**, 591-596 (1988).
4. Smith, R. L. *Nature* **224**, 351-352 (1969).
5. Gurnett, D. A. & Frank, L. A. *J. geophys. Res.* **77**, 172-190 (1972).
6. James, H. G. *J. geophys. Res.* **81**, 501-514 (1976).
7. Maeda, K. & Calvert, W. *J. geophys. Res.* (submitted).
8. Taylor, H. A. Jr, Grebowsky, J. M. & Cloutier, P. A. *J. geophys. Res.* **90**, 7415-7426 (1985).
9. Taylor, H. A. Jr, Grebowsky, J. M. & Cloutier, P. A. *J. geophys. Res.* **91**, 4599-4605 (1986).
10. Curtis, S. A., Brace, L. H., Nieman, H. B. & Scarf, F. L. *J. geophys. Res.* **90**, 6631-6636 (1985).
11. Temerin, M. C. et al. *J. geophys. Res.* **86**, 11278-11298 (1981).
12. Winglee, R. M. et al. *J. geophys. Res.* **93**, 14567-14590 (1988).

ACKNOWLEDGEMENTS. We thank A. Warnock for the microdensitometer traces, H. A. Taylor Jr for the Venus ion density data and Professor D. A. Gurnett for the DE-1 VLF wide-band data.

Evidence for cleavage of polymer chains by crack propagation

H. R. Brown*, V. R. Deline* & P. F. Green†

* IBM Almaden Research Center, 650 Harry Road, San Jose, California 95120-6099, USA

† Sandia National Laboratories, Albuquerque, New Mexico 87185-5800, USA

CRACK propagation in polymers is a topic of considerable importance to the technological application of these materials. In high-molecular-weight glassy polymers, crack propagation is normally assumed to require the breaking of covalent bonds in polymer chains that span the crack path, as opposed to chain slip and disentanglement. The fraction of such broken chains in a bulk sample would be very small, however, so that there is little direct evidence for this chain breakage¹. Interfaces between immiscible polymers are often very weak, but can be strengthened by the presence of a diblock copolymer (a polymer in which each molecule consists of a block of one polymer bonded to a block of a different polymer) for which each of the two blocks is miscible with one of the homopolymers². The diblock copolymer is thought to organize at the interface and so 'stitch' the homopolymers together. Here we show that crack propagation along the interface between the homopolymers involves breaking all the copolymer chains at a point in the region of the junction between the two blocks. Thus we demonstrate both that the block copolymer does indeed organize at the interface and that fracture involves the breaking of polymer chains. This information may suggest means by which the mechanical properties of polymer blends could be systematically improved.

Diblock copolymers can be made with one or both of the blocks deuterated. In our experiment we place partially or fully deuterated block copolymers at the interface between two homopolymers, join the homopolymers at elevated temperatures

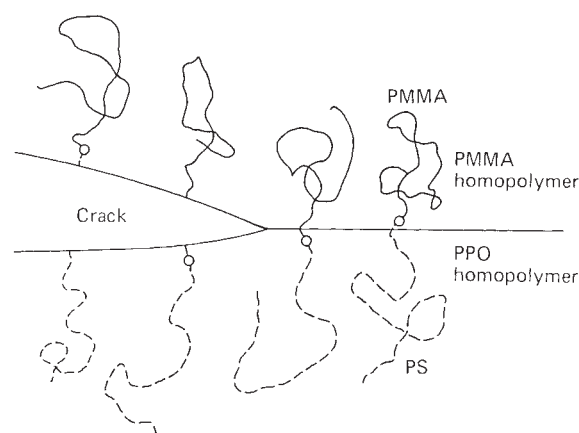


FIG. 1 A schematic view of the partly fractured interface with diblock-copolymer molecules present. The junction of the diblock is represented by a circle.

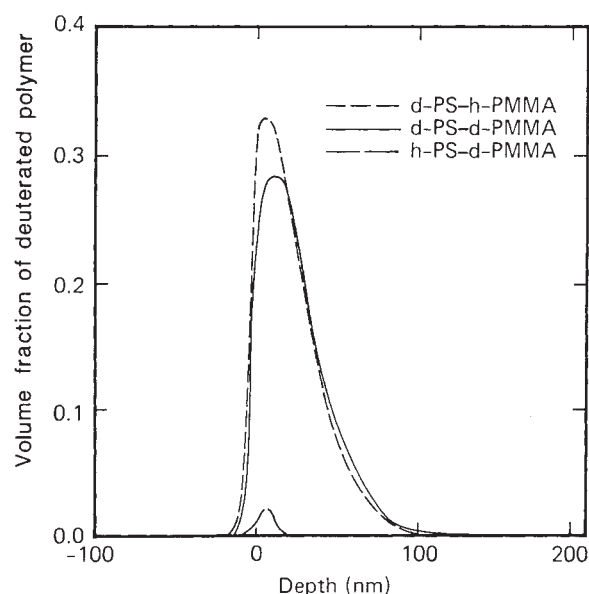


FIG. 2 SIMS deuterium profiles under the PPO fracture surface for the three block copolymers. Films of h-PS of 50-nm thickness were floated onto the sample from a water surface before the depth profiling. This was necessary to establish a constant etching rate before depth profiling started into the sample. The depth zero was chosen as the position of a thin gold layer between the h-PS cover layer and the sample.

and then fracture the sample along the interface at room temperature. We use secondary-ion mass spectrometry³ (SIMS) and forward-recoil spectroscopy^{4,5} (FRES) to measure the deuterium on the fracture surface. If the block copolymer breaks at its junction point, then deuterium will be seen on both surfaces when both blocks of the copolymer are deuterated but only on a single surface if one block is deuterated. The positions of the propagating crack and the diblock copolymer are shown schematically in Fig. 1.

Poly(2,6-dimethyl-1,4-phenylene oxide) (PPO) and poly(methylmethacrylate) (PMMA) are immiscible homopolymers, and the interface between them has a low interfacial toughness of $\sim 1.5 \text{ J m}^{-2}$. PPO is miscible with polystyrene (PS) so we expect PS-PMMA diblock copolymers to strengthen the interface between PPO and PMMA homopolymers. Such strengthening indeed exists; the presence of a 25-nm-thick layer of a

symmetric diblock of total relative molecular mass 280,000 (280K) gives an interface with a toughness of $\sim 15 \text{ J m}^{-2}$ (ref. 1), a value both significantly higher than that of the bare interface and considerably lower than that for cohesive failure of the homopolymers. The latter point is important as the weakness of the interface means that the fracture surface do not become highly deformed or crazed in the fracture process and so are suitable for analysis by SIMS and FRES.

The SIMS depth profiles for deuterium at the two fracture surfaces (Figs 2 and 3) show that an equal amount of deuterium exists on both surfaces when the (d-PS)-(d-PMMA) diblock is used (here d and h will indicate deuterated and non-deuterated polymers). For the (d-PS)-(h-PMMA) diblock, most of the deuterium is on the PPO fracture surface whereas most deuterium is on the PMMA surface for the (h-PS)-(d-PMMA) diblock. Clearly the diblock copolymer was at the interface and broke near its centre point when the specimen was fractured. These results were confirmed with the use of FRES (Fig. 4) which showed that the PPO half of the fractured sample contained PS but no observable PMMA from the diblock. (We note that the PS side of the block copolymer extends deeper into the PPO homopolymer than does the PMMA side of the copolymer into the PMMA homopolymer. This effect is caused by the favourable enthalpy of mixing between PS and PPO.)

Integration of the deuterium signal from SIMS profiles gives the proportion of diblock on the 'wrong' sides of the interface. The fraction of the PS on the PMMA side was 0.7% whereas 2.1% of the PMMA in the block copolymer was on the PPO side. If the distribution of fracture positions along the chain is assumed to be gaussian, one can estimate the width and position of the centre point of the distribution from a knowledge of gaussian integrals. For diblock chains of total relative molecular mass $\sim 300\text{K}$, the standard deviation of the distribution of fracture positions was found to be just 1.6% of the chain length (4.5K). The mean fracture position (centre of the gaussian) was 0.7% of the chain length into the PMMA from the junction point. These results show that 70% of the chains broke within a chain length of $\sim 9\text{K}$ centred at a point in the PMMA just 2K into the PMMA from the junction between the PS and PMMA. Such accurate bisection of the copolymer chains shows that the

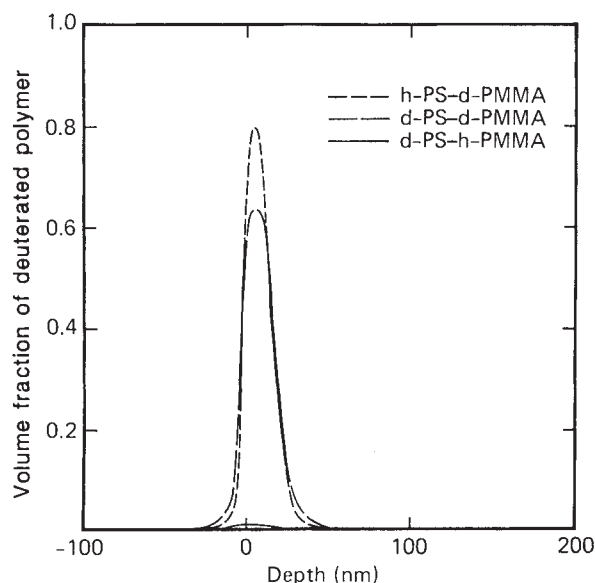


FIG. 3 SIMS deuterium depth profiles taken into the PMMA side of the fracture surface. An h-PS cover layer was used here as described for Fig. 2. Integration of the (d-PS)-(d-PMMA) curves for both fracture surfaces gave approximately the same amount of deuterium on each side.

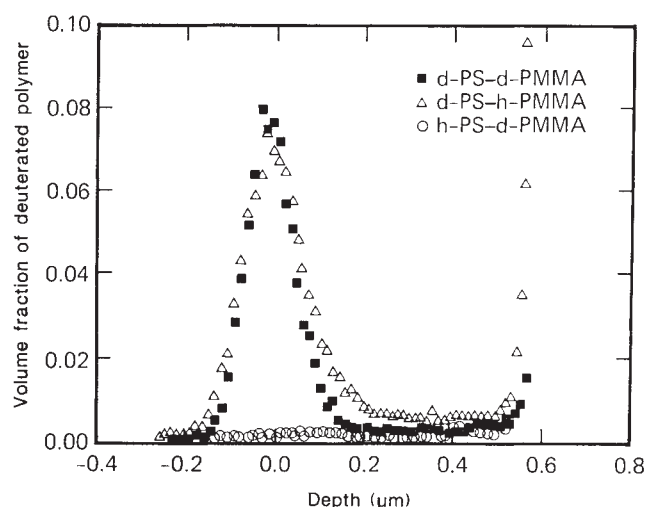


FIG. 4 Depth profiles obtained by FRES for deuterium from the PPO fracture surface. The limited depth resolution of the FRES technique makes it impossible to observe the shape of the profile.

block copolymer must be well organized at the interface with its junction accurately placed at the PPO/PMMA interface.

Although the entanglement spacing of a polymer is obtained from its rheological properties at temperatures above the glass transition temperature T_g , there is excellent evidence⁶ that the entanglement density controls the crazing and failure of a glassy polymer. Some recent models^{7,8} of crazing and failure in glassy polymers assume that the chains are loaded by stress transfer from adjacent chains at discrete entanglement points. This assumption implies that, in the copolymer, most failure should occur within a length corresponding to one entanglement spacing. The relative molecular mass between entanglements M_e is 9K (ref. 9) for PMMA. The M_e for PS in PPO is not known but, as the concentration of PS at the interface on the PPO side is $\geq 30\%$, it is probably not very different from the mean value of 7K measured¹⁰ for a 1:1 blend of PS and PPO. These values for M_e agree very well with the measured 9K length for 70% of the failures indicating that entanglement spacing does indeed control the length of the highly stressed chain at the crack tip.

Our experiment does not allow us to determine whether the copolymer chain breaks in more than one place. It is quite possible that the PMMA side of the copolymer forms a number of loops into the PPO. These loops, if they exist, must pull out during the crack propagation, perhaps by fracturing at their apexes, if relatively long. It would be, in principle, possible to detect this additional chain breakage by heating the broken samples above T_g and measuring the diffusion constant of the broken deuterated copolymer molecules into the matrix. This diffusion constant should depend on the relative molecular mass of the labelled chains. □

Received 12 July; accepted 10 August 1989.

1. Kausch, H. H. *Polymer Fracture* 2nd edn (Springer, Berlin, 1987).
2. Brown, H. R. *Macromolecules* **22**, 2859-2860 (1989).
3. Benninghoven, A., Rüdenauer, A. G. & Werner, H. W. *Secondary Ion Mass Spectrometry* (Wiley, New York, 1987).
4. Mills, P. J., Green, P. F., Palmstrom, C. J., Mayer, J. W. & Kramer, E. J. *Appl. Phys. Lett.* **45**, 957-959 (1984).
5. Green, P. F. & Doyle, B. L. *Characterization Techniques for Thin Polymer Films* (ed. Ho Ming Tong) (Wiley, New York, 1989).
6. Kramer, E. J. *Adv. Polym. Sci.* **52/3**, 1-56 (1983).
7. McLeish, T. C. B., Plummer, C. J. G. & Donald, A. M. *Polymer* (in the press).
8. Evans, K. E. *J. Polym. Sci., Polym. Phys. Edn* **25**, 353-368 (1987).
9. Ferry, J. D. *Viscoelastic Properties of Solid Polymers* 3rd edn (Wiley, New York, 1980).
10. Prest, W. M. Jr & Porter, R. S. *J. Polym. Sci., Polym. Phys. Edn* **10**, 1639-1655 (1972).

* Department of Chemistry and † Department of Pathology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Y6

Zeolites are open-framework structures, constructed from SiO_4^{4-} and AlO_4^{5-} tetrahedra, that usually contain channels and cavities and are of widespread interest because of their unique sorptive and catalytic properties¹⁻⁴. Although they are highly crystalline, they are usually microcrystalline with dimensions of only a few microns, precluding the straightforward use of single-crystal diffraction techniques and structural refinements must usually be attempted from more limited powder diffraction data. (We note that the information from powder diffraction data may be improved by the use of synchrotron X-ray sources, which makes it possible to work with much smaller crystals⁵, and Rietveld analysis.)

TABLE 1 Tetrahedral atom sites, their occupancies and connectivities for the asymmetric unit in zeolite ZSM-12

T-site	occupancy	connectivities
T_1	1	$2T_2:2T_3$
T_2	1	$2T_1:2T_4$
T_3	1	$2T_1:1T_5:1T_7$
T_4	1	$2T_2:1T_5:1T_6$
T_5	1	$1T_3:1T_4:2T_6$
T_6	1	$1T_4:2T_5:1T_7$
T_7	1	$1T_3:1T_6:2T_7$

Figure 1 shows one projection of the lattice structure of zeolite ZSM-12. The structure is approximately that originally reported¹⁶ although a recent refinement¹⁷ of synchrotron powder X-ray data established the unit-cell symmetry to be $C_{2/C}$ with a doubling of the c cell dimension to 24.3275 Å. The number of crystallographically inequivalent silicons in the unit cell is unchanged, there being seven tetrahedral (T) sites with equal occupancies as indicated in Fig. 1. This is reflected in the ^{29}Si magic-angle-spinning NMR spectrum which shows seven clearly separated resonances of equal intensity. Table 1 lists the connectivities predicted from the structure.

223

in Fig. 2. Clear connectivities are observed in the unsymmetrized plot; the truncation of the data in t_2 increases the signal intensity at the expense of resolution. The resonances may be assigned uniquely using only the expected connectivities presented in Table 1 as a guide (Fig. 2). Alternatively, T_5 may be unambiguously assigned as the resonance at $\delta = -112.3$ p.p.m. as it decreases significantly in intensity relative to the other signals if the recycle times used are too short, indicating that it has a much longer spin-lattice relaxation time. A major contribution to the relaxation mechanism is ^{29}Si dipolar interactions with molecular oxygen and T_5 is the only site that is not on the surface of a channel wall. From this assignment one can proceed to assign the others. Both of these approaches yield the labelling of the cross-peaks given in Fig. 2.

When the number of points in t_2 is increased to 450, the two-dimensional plot shows the same connectivities but with a lower signal-to-noise ratio (Fig. 3a). More interestingly, doublet splittings are observed for the cross-peaks in frequency F_2 (the limited resolution in F_1 precludes their observation). Figure 3b shows plots of rows from this two-dimensional experiment corresponding to the maxima in the diagonal peaks where the fine structure is clearly observable. The splittings are all 9–16 Hz, but these are approximate values only because the real digital resolution before zero-filling is only ~ 3 Hz per point and a power calculation has been used in the data processing. These data may be compared with the ^{29}Si -O- ^{29}Si couplings observed¹⁹ in alkaline silicate solutions enriched in ^{29}Si . The values range from 3 to 17 Hz with most couplings < 10 Hz but many of the silicate species in solution have high concentrations of three-membered rings and do not relate directly to the local structures in zeolites. Silicon atoms in four- and five-membered rings in

the species show couplings of the order of 10 Hz, approximately the values observed in the present work and we feel that the present data represent a direct observation of scalar ^{29}Si -O- ^{29}Si couplings in the solid state.

The ability to obtain three-dimensional lattice connectivities in zeolites using natural-abundance samples greatly extends the potential of solid-state NMR techniques in this area and when combined with information from synchrotron X-ray powder diffraction should greatly assist in the solution of unknown structures. ^{29}Si COSY experiments such as these described above and also ^{29}Si INADEQUATE experiments (another pulse sequence that can establish connectivity patterns) have been applied successfully to a number of different zeolite structures (C.A.F., Y.F., H.G., H. Grondy and G.T.K., manuscript in preparation). □

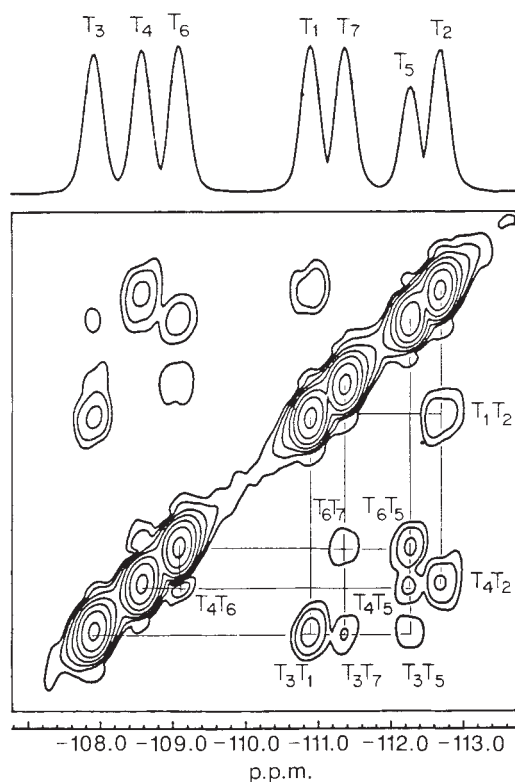


FIG. 2 Contour plot of a COSY experiment in ZSM-12 with the projection in the F_2 dimension shown on top. The temperature was 300 K and 64 experiments were carried out with 160 scans in each experiment. A sweep-width of 1,200 Hz, a fixed delay of 5 ms and 256 real data points were used (other channels are zero-filled). Sine-bell-squared apodization and magnitude calculation were used for the data processing.

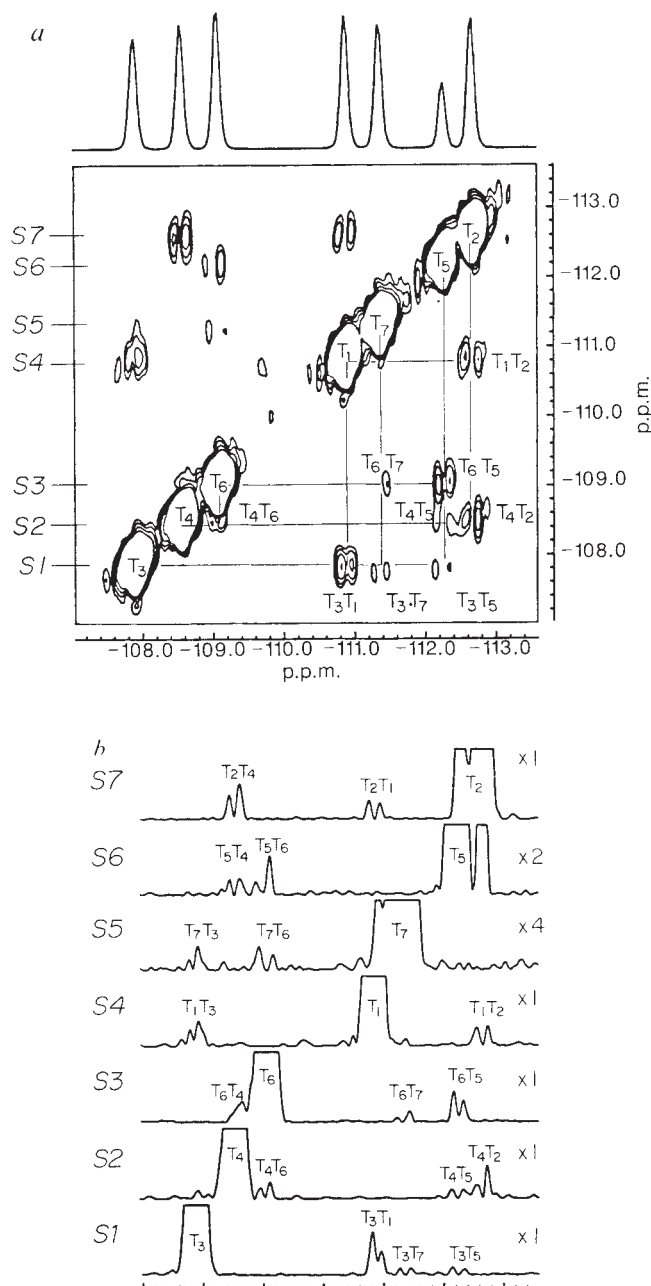


FIG. 3 a, Contour plot, with projection in F_2 , of COSY experiment on ZSM-12. The same conditions as those in Fig. 2 were used except that 80 scans were taken in each experiment. 450 real data points and power calculation were used in the data analysis. S1-S7 mark the rows of fixed frequency F_1 for the frequency F_2 spectra shown in b.

Received 24 July; accepted 14 August 1989.

1. Breck, D. W. *Zeolite Molecular Sieves* (Wiley Interscience, New York, 1974).
2. Smith, J. V. *American Chemical Society Monographs* (ed. Rabo, J.) **171**, 3-79 (1976).
3. Barrer, R. M. *Zeolites and Clay Minerals as Sorbents and Molecular Sieves* (Academic, London, 1978).
4. Holderich, W., Hesse, M. & Nümann, F. *Angew. Chem.* **27**, 226-246 (1988).
5. Eisenberger, P., Newman, J. B., Leonowicz, M. E. & Vaughan, D. E. W. *Nature* **309**, 45-47 (1984).
6. Fyfe, C. A. *Solid State NMR for Chemists* (CFC Press, Guelph, 1984).
7. Engelhardt, G. & Michel, D. *High Resolution Solid-State NMR of Silicates and Zeolites* (Wiley, New York, 1987).
8. Fyfe, C. A., Gobbi, G. C., Murphy, W. J., Ozubko, R. S. & Slack, D. A. *J. Am. Chem. Soc.* **106**, 4425-4438 (1984).
9. Benn, R. & Günther, H. *Modern Pulse Methods in High Resolution NMR Spectroscopy* *Angew. Chem. int. Edn engl.* **22**, 350-380 (1983).
10. Bax, A. *Two Dimensional Nuclear Magnetic Resonance in Liquids* (Delft University Press, 1982).
11. Derome, A. E. *Modern NMR Techniques for Chemistry Research* (Pergamon, Oxford, 1987).
12. Sanders, J. & Hunter, B. *Modern NMR Spectroscopy, A Guide for Chemists* (Oxford University Press, 1987).
13. Kessler, H., Gehrke, M. & Griesinger, C. *Angew. Chem. int. Edn engl.* **27**, 490-536 (1988).
14. Benn, R., Grondy, H., Brevard, C. & Pagelot, A. *JCS chem. Commun.* 102-103 (1988).
15. Fyfe, C. A., Gies, H. & Feng, Y. *JCS chem. Commun.* (in the press).
16. LaPierre, R. B. *et al. Zeolites* **5**, 346-348 (1985).
17. Fyfe, C. A., Gies, H., Kokotailo, G. T., Marler, B. & Cox, D. E. *J. phys. Chem.* (submitted).
18. Muller, L., Kumar, A. & Ernst, R. R. *J. chem. Phys.* **63**, 5490-5491 (1975).
19. Harris, R. K. & Knight, C. T. *G. JCS Faraday Trans. II*, **79**, 1539-1561 (1983).

ACKNOWLEDGEMENTS. We acknowledge the financial assistance of Natural Science and Engineering Research Council of Canada (C.A.F.) and the Alexander von Humboldt Foundation (H.G., G.T.K.). C.A.F. also acknowledges the award of a Killam Research Fellowship by the Canada Council and Y.F. the award of a University Graduate Scholarship. H.G. is on leave from Mineralogisches Institut der Christian Albrechts Universität (Kiel).

An optical yield that increases with temperature in a photochemically induced enantiomeric isomerization

Yoshihisa Inoue, Taizo Yokoyama, Noritsugu Yamasaki & Akira Tai

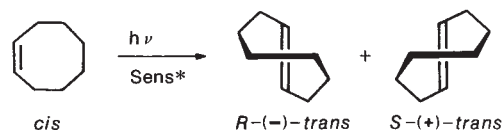
Basic Research Laboratory, Himeji Institute of Technology, 2167 Shosha, Himeji, Hyogo 671-22, Japan

TRANSFER of chirality and multiplication of chiral molecules are key processes in the chemical and biochemical transformations of natural and synthetic compounds. For entropic reasons, structural immobilization in enzymatic and catalytic processes and the use of low temperatures should generally enhance the optical yield (preference for one enantiomer) in chiral reactions. Studies of the temperature dependence of optical yield in chemical and biochemical processes have, however, been sporadic¹⁻¹⁰. Here we report a systematic study of the temperature dependence of the photosensitized enantiodifferentiating isomerization of a simple alkene. We find that above a critical temperature, characteristic of the photosensitizer used, the optical yield increases with increasing temperature, apparently conflicting with the widely accepted view that lower temperatures favour a higher optical yield. Thus both enantiomers may be produced with high efficiency using a single chiral source. This implies that transfer and multiplication of chirality in natural systems, an important aspect of the development of prebiotic organic molecules, may be effected rather more simply than has hitherto been supposed.

Efficient photosensitized enantio-differentiation (producing one enantiomer preferentially) has proved a difficult process to achieve. Despite considerable efforts over two decades¹¹⁻¹⁷, no chiral photosensitization system has exceeded the optical yield of 7% reported in ref. 12. This has not been considered surprising because the steric interaction between sensitizer and substrate is not sufficiently strong and long-lived in most photoreactions. Photochemical reactions are more amenable than thermal reactions to studies of the temperature dependence of the optical yield because in the latter case the reaction mechanism may change with temperature.

We have demonstrated^{15,16} that the (-)-menthylbenzoate- or

isophthalate-sensitized *cis-trans* photoisomerization of *cis*-cyclooctene proceeds through a singlet exciplex and yields optically active *R*-(-)-*trans*-cyclooctene in 2-4% enantiomeric excess (ee) at room temperature.



Here we use (-)-menthyl and/or (-)-bornyl esters of benzoic, phthalic and pyromellitic acid as chiral sensitizers. To explore the temperature effect on the optical yield, the photosensitized enantio-differentiating isomerizations were performed at temperatures ranging from -88 to +50 °C. The solutions (300 ml of pentane, 0.2M *cis*-cyclooctene and 5-10 mM chiral sensitizer) were contained in an annular quartz vessel that surrounded a 30-W mercury-resonance lamp fitted with a Vycor sleeve, which eliminates the 185-nm resonance line from the lamp. The 254-nm irradiations were carried out under an argon atmosphere, in a thermostated methanol bath (± 0.5 -1.0 °C), for a period sufficient to establish a photostationary state. After irradiation, the photolysate was extracted¹⁶ with aqueous silver nitrate to isolate the *trans* isomer. The optical yields were determined by comparing the optical rotation of each product with the reported value $[\alpha]_D^{25} = -426^\circ$ (*c* (grammes of sample per 100 ml of solvent) = 0.41, CH₂Cl₂)¹⁸.

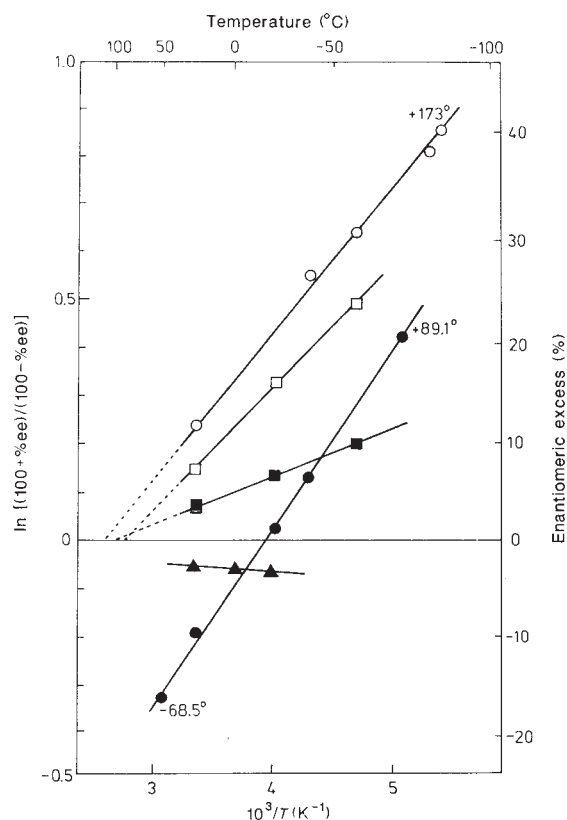


FIG. 1 Temperature dependence of optical yield: $\ln[(100 + \%ee)/(100 - \%ee)]$ as a function of reciprocal temperature in the enantio-differentiating photoisomerization of *cis*-cyclooctene sensitized by (-)-menthyl benzoate ($\blacktriangle$), di-(-)-menthyl ($\blacksquare$) and di-(-)-bornyl phthalate ($\bullet$), and tetra-(-)-menthyl ($\circ$) and tetra-(-)-bornyl pyromellitate ($\circ$) in pentane solution; numbers in the plot show the optical rotations of the isolated *trans*-cyclooctene, $[\alpha]_D^{25}$ (*c* 1.0-3.4, CH₂Cl₂); accuracy in temperature ± 0.5 -1.0 °C; estimated error in %ee is $\pm 0.2\%$; reproducibility of %ee was tested with menthyl benzoate and dimethyl phthalate at 25 °C—on the scale of this figure the points are almost superimposed.

In Fig. 1, we plot $\ln[(100 + \%ee)/(100 - \%ee)]$, where $\%ee$ has the same sign as the optical rotation, as a function of the reciprocal of irradiation temperature. In the case of menthyl benzoate sensitization, the optical yield decreases linearly with decreasing inverse irradiation temperature. The extrapolation to infinite temperature predicts a optical yield of virtually zero. A linear relationship is also obtained for reactions in which the sensitizers are the chiral phthalate and pyromellitate. Here, however, the zero-optical-yield line is crossed at finite temperatures (Fig. 1). In the case of methyl pyromellitate sensitization, the preferred chirality changes at -19°C . For the other phthalates and pyromellitate sensitizers, similar changes are anticipated at 90 – 125°C , just above the experimental temperature range. Below this critical temperature, which we tentatively name the iso-enantio-differentiating or simply the equipodal temperature T_0 , the photoisomerization yields more *S*-(+)-*trans* isomer and the optical yield increases with decreasing temperature. The highest enantiomeric excess that we found was 40.6% for the tetrabornyl pyromellitate sensitization at -88°C . Extrapolation predicts an enantiomeric excess of 100% at -246 and -243°C for bornyl and methyl pyromellitate, respectively. Beyond T_0 the *R*-enantiomer predominates and the optical yield increases as the irradiation temperature increases, approaching the maximum enantiomeric excesses of 12–63% at infinite temperature.

This remarkable temperature dependence of the optical yield may be interpreted in terms of temperature-dependent populations of the diastereomeric exciplexes of the excited pyromellitates, which possess bulky substituents at the *ortho* positions, and the cyclooctene. We infer that the exciplex leading to *S*-(+)-configuration is predominant at low temperatures,

whereas the other prevails at high temperatures and leads to an excess of *R*-(-)-configurations. We suggest that the steric interaction between adjacent bulky chiral alcohol moieties generates the diastereomeric exciplexes because only the *ortho* dicarboxylates like phthalate and pyromellitate exhibit this unusual temperature dependence, and because there is no indication of the ground-state diastereomeric conformers over the temperature range of -90 to $+25^\circ\text{C}$ in the ^1H NMR (in fully deuterated acetone at 400 MHz) and circular dichroism spectra (in pentane) of tetra-(-)-menthyl pyromellitate. □

Received 16 May; accepted 9 August 1989.

1. Morrison, J. D. & Mosher, H. S. *Asymmetric Organic Reactions* (Prentice-Hall, Englewood Cliffs, 1971).
2. Izumi, Y. & Tai, A. *Stereo-differentiating Reactions* (Kodansha-Academic, Tokyo, 1977).
3. Rety, J. & Robinson, J. A. *Stereospecificity in Organic Chemistry and Enzymology* (Verlag Chemie, Weinheim, FRG, 1982).
4. Morrison, J. D. (ed.) *Asymmetric Synthesis* Vols 1–5 (Academic, New York, 1983–1984).
5. Coppola, G. M. & Schuster, H. F. (eds) *Asymmetric Synthesis* (Wiley-Interscience, New York, 1987).
6. Harada, K. & Yoshida, T. *JCS, chem. Commun.* 1071 (1970); *J. org. Chem.* **37**, 4366–4370 (1972).
7. Harada, K. & Kataoka, Y. *Chem. Lett.* 791–794 (1978).
8. Ojima, J., Kogure, T. & Toda, N. *J. org. Chem.* **45**, 4728–4739 (1980).
9. Sinou, D. *Tetrahedron Lett.* **22**, 2987–2990 (1981).
10. Landis, C. R. & Halpern, J. J. *Am. chem. Soc.* **109**, 1746–1754 (1987).
11. Rau, H. *Chem. Rev.* **83**, 535–547 (1983).
12. Hammond, G. S. & Cole, R. S. *J. Am. chem. Soc.* **87**, 3256–3257 (1965).
13. Ouannes, C., Beugelmans, R. & Roussi, G. *J. Am. chem. Soc.* **95**, 8472–8474 (1972).
14. Balavoine, G., Juge, S. & Kagan, H. B. *Tetrahedron Lett.* 4159–4162 (1973).
15. Inoue, Y., Kunitomi, Y., Takamuku, S. & Sakurai, H. *JCS, chem. Commun.* 1024–1025 (1978).
16. Inoue, Y., Takamuku, S., Kunitomi, Y. & Sakurai, H. *JCS, Perkin Trans. II*, 1672–1677 (1980).
17. Goto, S., Takamuku, S., Sakurai, H., Inoue, Y. & Hakushi, T. *JCS, Perkin Trans. II*, 1678–1681 (1980).
18. Cope, A. C., Ganellin, C. R., Johnson, H. W. Jr, Van Auken, T. V. & Wirkler, H. J. S. *J. Am. chem. Soc.* **85**, 3276–3279 (1963).

ACKNOWLEDGEMENTS. We thank Professor T. Takagi for the use of his circular dichroism instrument. We acknowledge the financial support of the Ministry of Science, Culture and Education of Japan.

Amyloid β -protein deposition in tissues other than brain in Alzheimer's disease

Catharine L. Joachim, Hiroshi Mori & Dennis J. Selkoe

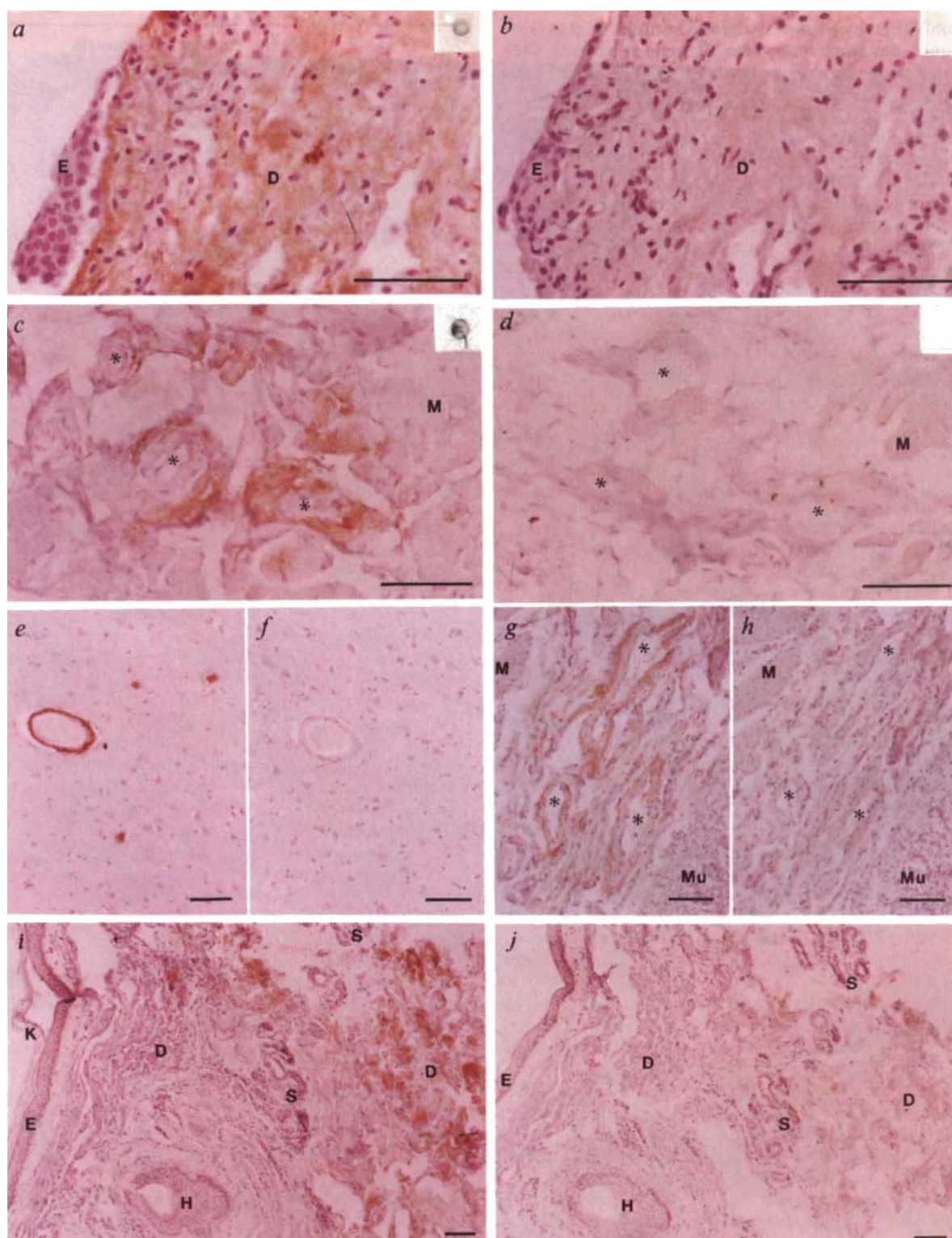
Department of Neurology (Neuroscience), Harvard Medical School and Center for Neurologic Diseases, Department of Medicine (Neurology), Brigham and Women's Hospital, Boston, Massachusetts 02115, USA

ALZHEIMER'S disease is the most common cause of progressive intellectual failure in aged humans. The filamentous brain lesions which define the disease occur within neurons (neurofibrillary tangles), in extracellular cerebral deposits (amyloid plaques) and in meningeal blood vessels (amyloid angiopathy)^{1,2}. They are found in lesser numbers in the brains of virtually all old humans¹. A protein with a relative molecular mass (M_r) of $\sim 4,000$, designated amyloid β -protein or amyloid A4 protein, is the subunit of the vascular and plaque amyloid filaments in individuals with Alzheimer's disease^{3–5}, normal ageing⁶ and trisomy 21 (Down's syndrome)⁷. The amyloid β -protein is a small fragment of a membrane-associated glycoprotein^{8–13}, encoded by a gene on human chromosome 21 which is telomeric to a genetic defect that causes at least some cases of familial Alzheimer's disease^{14,15}. Until now, the pathological lesions of the disease have been found only in the brain, although reports of phenotypic abnormalities in non-neural tissues^{16–20} have suggested that Alzheimer's disease may be a widespread, systemic disorder. Here we report the detection of amyloid β -protein deposits in non-neural tissues and blood vessels of Alzheimer's disease patients, including skin, subcutaneous tissue and intestine. The protein was also present in non-neural tissues in a proportion of aged, normal subjects. Our findings indicate that a principal feature of the disease process is expressed subclinically in tissues other than brain. The occurrence

of amyloid β -protein deposits in multiple tissues suggests that the protein may be produced locally in numerous organs or may, as in other human amyloidoses, be derived from a common circulating precursor. These observations affect the rationale for many experiments analysing the amyloid β -protein precursor and its messenger RNAs in Alzheimer's disease brain tissue and have major implications for the pathogenesis and treatment of the disease.

Amorphous, non-filamentous deposits of amyloid β -protein (β AP) without surrounding neuronal or glial alteration have recently been demonstrated in the brains of Alzheimer's disease patients and younger subjects with trisomy 21^{21–24}. Because such 'preamyloid' deposits may represent a very early structural change in the brain, the question of the origin of amyloid deposits in Alzheimer's disease assumes added importance. We searched intensively for β AP deposits in tissues other than brain because of evidence that proteins present in cerebral plaques and vascular amyloid could derive in part from a circulating source^{25–30}. We initially examined perianal skin taken at autopsy from a 78-year-old man with typical Alzheimer's disease and severe brain lesions. An antiserum to a synthetic peptide of residues 1–38 of β AP produced specific labelling of amorphous material deposited multifocally in the connective tissue and periarterial spaces of the dermis and subcutaneous tissue

FIG. 1 Immunocytochemical detection of β AP in non-neural tissues of Alzheimer's disease patients. *a*, Section of non-keratinized perianal skin from a 78-year-old male with neuropathologically typical Alzheimer's disease (A85-196 in Table 2) reacted with control-absorbed antiserum A to native β AP purified from Alzheimer's cortex⁵. E, epidermis; D, dermis. Inset, dot immunoblot of amyloid-plaque core fraction stained simultaneously. *b*, Adjacent section to *a* (and corresponding dot blot of plaques cores) reacted with antiserum A after absorption with plaque cores. *c*, Subcutaneous tissue (case A85-196) reacted with antiserum Y to synthetic β AP_{1–38}. Note circumferential periarterial locus of β AP-reactive material. Asterisks indicate lumens of arterioles; M, muscle fibres. Inset, dot of peptide β AP_{1–38} stained simultaneously. *d*, Adjacent section to *c* (and corresponding dot) ▶



► reacted with antiserum Y after absorption with βAP_{1-38} . Absorption with an unrelated synthetic peptide ($\beta\text{APP}_{258-267}$; ref. 8) did not alter the staining. *e*, Alzheimer cerebral cortex reacted with antiserum A (control absorbed). Note characteristic staining of three amyloid plaque cores and amyloidotic arteriole. *f*, Adjacent section to *e* reacted with antiserum A absorbed with plaque cores. (Sections *e* and *f* were stained simultaneously with sections *i* and *j*) *g*, Section of intestine from an 87-year-old female with typical Alzheimer's disease (A88-159 in Table 2) reacted with antiserum A (control absorbed). Note vascular staining in submucosal connective tissue. Asterisks indicate lumens of vessels; Mu, mucosa; M, smooth muscle fibres. Other βAP antisera produced a slightly different pattern of vessel wall staining. *h*, Adjacent section to *g* reacted with antiserum A absorbed with plaque cores. (Sections *g* and *h* were stained simultaneously with sections *a* and *b*). *i*, Biopsied skin (30-min formalin fixation) from an 85-year-old male with

clinically typical Alzheimer's disease (X1330 in Table 2) reacted with antiserum A (control absorbed). Note strong βAP -immunoreactivity found multifocally in reticular dermis. E, epidermis; D, dermis; K, keratin; H, hair follicle; S, sweat glands. *j*, Adjacent section to *i* reacted with antiserum A absorbed with plaque cores. (Compare *i* and *j* with *e* and *f*). Bars, 100 μm .

METHODS. Six-micron sections of formalin-fixed, paraffin-embedded tissues were stained with primary antibodies (1:250 for A or 1:500 for Y) for 12 h (4 °C) and reacted with biotinylated goat anti-rabbit IgG in an avidin/biotin peroxidase system (Vector Labs., Burlingame, California) according to standard protocols. Absorptions were performed by incubating (12 h, 4 °C) an antiserum with purified amyloid-plaque cores⁵ (20 μg plaque-core protein per 1 μl antiserum) or with identically prepared control brain fractions (see text). For antiserum Y, synthetic βAP_{1-38} was used as absorbent.

which was inhibited by peptide. Adjacent sections of skin from this man, and skin from nine other Alzheimer patients were then examined with a panel of well-characterized antibodies to purified native β AP^{5,31,32}, synthetic β AP or unrelated proteins (Table 1). All four β AP antibodies that were tested produced selective, antigen-specific labelling of dermal and/or subcutaneous deposits (Fig. 1).

To determine the specificity of the immunolabelling, antigen-absorbed and 'control-absorbed' aliquots of each antiserum were reacted simultaneously with Alzheimer brain tissue and skin, and dot blots of the antigen (Fig. 1). For antiserum A, for example, partially purified SDS-extracted plaque cores⁵ were used as the absorbent; a control absorption used an identically prepared fraction from aged normal brain tissue which contained the contaminating particles of plaque-core fractions (lipofuscin, collagen and microvessel fragments). In each such experiment, the plaque-core absorption significantly diminished or abolished the staining of plaque and vascular amyloid in Alzheimer's disease brain tissue, the dermal and subdermal amorphous deposits, and the antigen on dot blots, simultaneously (Fig. 1*b, d, f* and *j*). The control absorptions produced no change (Fig. 1*a, c, e* and *i*). Absorptions of unrelated antisera (P, DJ and G in Table 1) with the same plaque core fractions did not alter their immunoreactivities. Pre-immune sera, secondary antibodies alone and various antibodies (Table 1) to purified paired helical filaments³³, tau protein³², glial fibrillary acidic protein³⁴, transthyretin (prealbumin) or the amyloid AA protein^{35,36} all failed to label the skin deposits.

Because some β AP deposition in cerebral tissues and vessels occurs commonly during normal ageing, we examined samples of skin, subcutaneous tissue or intestine from 41 human subjects: 11 with Alzheimer's, 26 without Alzheimer's and 4 with Down's syndrome (Table 2). In all but four of the subjects, autopsies had established precise neuropathological diagnoses. Eight of the eleven cases of Alzheimer's disease showed definite β AP-reactive non-neural deposits and two showed slight, equivocal reactivity. By contrast, only 3 of 26 non-Alzheimer subjects showed definite non-neural β AP reactivity; all of these were ≥ 77 -years-old. Of four Down's syndrome skin samples, two were β AP-reactive and two were equivocal. In addition to autopsy-proven cases, we examined fresh punch biopsies of forearm skin from one Alzheimer subject and one normal subject (Table 2): the biopsy from the Alzheimer subject (age 85) showed specific β AP-staining (Fig. 1*i, j*), whereas that from the normal subject (age 64) did not.

Amyloid β -protein-immunoreactive deposits were located in the dermis in a patchy distribution beneath the epidermal/dermal junction, sometimes near small blood vessels or glandular elements. The stained material seemed to be localized extracellularly among connective tissue fibres in the reticular dermis (Fig. 1*a, i*). Some deposits were found circumferentially around small vessels, particularly arterioles. Most vessels and other skin structures (sweat and sebaceous glands, hair follicles, hair shafts, smooth muscle, epidermis and keratin) were unstained (Fig. 1*i*), further showing the specificity of the reaction. In subcutaneous tissue (Fig. 1*c*) and the submucosal tissue of the intestine (Fig. 1*g*), β AP-reactivity was found in and around microvessels and in perivascular connective tissue, apparently extracellular. Deposits of β AP were most easily visualized in the intestine, because they often occurred in a distinct vascular locus resembling that found in the brain and meninges. Almost all of the dermal deposits we observed had an amorphous, non-fibrillar appearance, perhaps analogous to the diffuse β AP deposits found in abundance in Alzheimer brains and the brains of some patients with hereditary cerebrovascular β -amyloidosis of Dutch type³⁷. The non-neural deposits lost their reactivity with antibodies to synthetic β AP (Y in Table 1) when sections were pretreated with formic acid, a reagent that can enhance the staining of some brain β AP deposits³⁸. The deposits in skin were not detected by the stains Congo red or thioflavin S.

TABLE 1 Summary of antibodies

Antiserum	Immunogen	Reference
A	β AP ($M_r \sim 4,000$) purified by HPLC from Alzheimer cortex	5, 32
C	β AP ($M_r \sim 4,000$) purified by HPLC from Alzheimer cortex	5, 32
F	β AP ($M_r \sim 4,000$) purified by HPLC from Alzheimer meninges	31
Y	Synthetic β AP ₁₋₃₈ peptide	this report
P	Isolated paired helical filaments from Alzheimer cortex	33
DJ	Heat-stable microtubule-associated proteins (including tau) from human fetal brain	32
G	Glial fibrillary acidic protein	34
T	Human transthyretin	Calbiochem
AA	Isolated amyloid AA fibrils	35
Monoclonal antibody CB7	Isolated AA amyloid fibrils	36

References cited provide the characterization and typical dilutions used for each antiserum.

Our results provide the first demonstration that β AP, which is invariably and progressively deposited in the brain in Alzheimer's disease, is also deposited in extracerebral tissues. Four specific antibodies to native or synthetic β AP detect the tissue deposits before but not after β AP antigen absorption; staining of β -amyloid in the brain is simultaneously abolished. So far, antisera to proteins other than β AP have not labelled the non-neural deposits. Presumably, β AP was not detected in tissues other than brain previously because: (1) non-neural deposits (like many diffuse β AP deposits in the brain) are weakly reactive or not reactive with classical amyloid stains like Congo red and thioflavin; (2) the skin, which shows patchy, amorphous β AP immunoreactivity, has never been thought to be a likely site for β -amyloidosis in Alzheimer's disease, and (3) only antibodies to synthetic β AP, which detect non-neural deposits much less sensitively than our antisera to native β AP^{5,31}, were used. The presence of β AP in many of the skin sections we examined would have been difficult to establish if we had relied solely on synthetic β AP antibodies.

Our findings have several implications. First, β AP deposition, believed to be a very early change in Alzheimer's disease and Down's syndrome brains²¹⁻²³, can occur in multiple sites throughout the body both in Alzheimer's and during normal ageing. This indicates that β AP deposition does not require preceding neuronal injury. Non-fibrillar forms of β AP are detectable in several tissues, but local cellular changes which are sufficient to cause clinical dysfunction are apparently associated only with some deposits in certain regions of the brain. Second, our findings may indicate multiple sites of β AP production by local cells in skin, intestine and other organs, including some cells in the brain. Alternatively, the presence of β AP in widespread non-neural tissues, particularly in and around arterioles, and its frequent perivascular deposition in meninges and the brain, are consistent with the hypothesis that β AP occurs in some circulating form and can reach tissues by means of the blood. No circulating form of β AP has yet been defined, and a mechanism whereby the hydrophobic, putatively transmembrane domain comprising β AP could circulate in the blood needs to be sought. Third, some of the phenotypic abnormalities previously reported in non-neural tissues of aged and Alzheimer's disease patients, particularly in skin-derived cells, could relate in part to the progressive deposition of β AP in these peripheral tissues. For example, a comparison of the properties of fibroblasts harvested from Alzheimer's skin that is either rich in or devoid of β AP deposits could be of interest.

TABLE 2 β AP immunoreactivity in non-neural tissue samples from 41 human subjects

Subject	Age (yr) sex	Neurological diagnosis	Neuropathology	β AP Immunoreactivity	
				Skin	Colon
Alzheimer's disease					
Skin					
A88-46	89 F	AD (>5)*, remote CVA	AD, slight AA, remote CVA	+	
X1330	85 M	AD (6)*	[living patient]	+	
A88-77	84 M	AD (14)*	AD, moderate AA	+	
A88-218	82 M	AD (2)	AD, slight AA, acute CVA	+	
A85-196	78 M	AD (7)*	AD, marked AA	+	
A86-55	77 F	AD/PD (1)*	AD/PD†, moderate AA	+	
A87-237	76 F	AD (7)*	AD, moderate AA	—	
A87-48	74 F	AD (4)*	AD, slight AA	+	
A89-10	71 M	AD (5)*	AD, moderate AA	+	
A89-14	62 F	AD (8)*	AD, moderate AA	±	
Intestine					
A88-46	89 F	AD (>5)*, remote CVA	AD, slight AA, remote CVA		±
A88-159	87 F	AD (>5)*	AD, marked AA		+
Aged diseased					
Skin					
A87-144	83 F	Pd, dementia (5)*	No PD, slight SP, no AA	—	
A88-233	82 F	PD (8)*, dementia (1)*	Nigral/pallidal degeneration, no SP slight AA	—	
A88-52	81 M	Remote poliomyelitis	Remote polio, no SP, no AA	+	
A88-130	79 M	Remote CVA	Remote CVA, slight SP, no AA	—	
A89-40	78 M	Mental retardation	Remote contusion, no SP, no AA	+	
A88-125	77 F	Remote CVA	Remote CVA, moderate SP, slight AA	—	
Intestine					
A88-125	77 F	Remote CVA	Remote CVA, moderate SP, slight AA		+
A88-194	62 F	Carcinoma	Metastatic carcinoma, no SP, no AA		—
Aged normal					
Skin					
A87-45	80 M	Normal	Rare SP, slight AA	±	
A87-147	76 M	Normal	No SP, moderate AA	—	
A87-302	72 F	Normal	No SP, no AA	—	
A88-347	72 M	Normal	No SP, slight AA	±	
X1362	64 M	Normal	(living patient)	—	
Non-aged normal					
Skin					
A87-268	61 F	Normal	Normal	—	
A89-3	60 M	Brain tumour	Glioblastoma	—	
A88-376	56 F	Normal	Normal	—	
A88-259	55 F	Normal	ND	—	
A88-258	49 M	Normal	Normal	—	
A88-358	45 M	Normal	Normal	—	
A88-367	45 F	Normal	Normal	—	
A83-256	42 M	Spinal cord trauma	Traumatic myelopathy	—	
A88-280	35 M	Brain tumour	Glioblastoma	—	
A88-368	32 M	Normal	Normal	—	
A88-300	30 M	Normal	ND	—	
A88-159	Fetus	Prematurity	Germinal matrix haemorrhage	—	
Intestine					
A88-183	38 F	Normal	Normal		—
A88-160	24 F	Normal	Normal		—
Down's syndrome					
Skin					
A80-40	44 F	Down's syndrome	Moderate SP, slight AA	+	
A85-140	38 M	Down's syndrome	Moderate SP, no AA	±	
A86-98	36 F	Down's syndrome	Moderate SP, slight AA	±	
A77-61	25 M	Down's syndrome	Moderate SP, no AA	+	

M, male; F, female AD, Alzheimer's disease; PD, Parkinson's disease; AA, amyloid angiopathy; CVA, cerebrovascular accident; SP, neocortical senile plaques; +, definite, specific staining; —, no staining; ±, slight or equivocal staining. ND, not determined. Skin samples (with subcutaneous tissue) were taken at autopsy, usually from the abdomen (except X1330 and X1362, which were 3-mm forearm punch biopsies). Skin samples from autopsy measured 14.3 ± 5.8 mm in length (mean $\pm$ s.d.) and 7.3 ± 5.0 mm in depth; sizes did not differ between Alzheimer's disease and controls. Each skin or colon sample was tested for specific β AP reactivity (see text and Fig. 1 legend) in 3–10 distinct immunostaining experiments carried out on different days. All stained sections (with simultaneous controls; see text) were examined and scored independently by each of the three investigators. In all cases but two (A89-14 and A88-347), all three investigators obtained the same score; the two exceptional cases each received scores of ±, ± and —.

* Approximate duration of disease (years).

† Typical neuropathological findings of both AD and PD.

A fourth implication of our findings is that the detection and quantitation of β AP in skin or another peripheral tissue might ultimately prove useful in confirming a clinical diagnosis of Alzheimer's disease and in following the course of the illness. Only a much larger prospective study of optimally preserved skin samples from patients with Alzheimer's disease and from aged normal subjects that have been biopsied and autopsied will clarify this issue. As expected, some aged normal humans were found to have β AP-reactive material in non-neural tissues, just as occurs in the brain. Amorphous deposits of cystatin C have recently been found in dermis and other non-neural tissues of Icelandic patients with hereditary cystatin C cerebrovascular amyloidosis³⁹. Some apparent correlation between the degrees of skin- and cerebrovascular-cystatin C deposition has been observed in this late-onset, autosomal dominant amyloidosis of the brain (H. Blöndal, G. Guomundsson & E. Benedikz, personal communication).

Finally, if the perivascular β AP deposits in multiple tissues of Alzheimer's disease and aged, normal subjects arise from a circulating form of the molecule, then this could have potentially important implications for the design of novel therapeutic approaches to prevent the progressive deposition of β -amyloid in the brain. □

Received 10 April; accepted 8 August 1989.

1. Tomlinson, B. E. & Corsellis, J. A. N. in *Greenfield's Neuropathology* (eds Adams, J. H., Corsellis, J. A. N. & Duchon, L. W.) 951–1025 (Arnold, London, 1984).
2. Anderton, B. H. *Nature* **325**, 658–659 (1987).
3. Glenner, G. G. & Wong, C. W. *Biochem. biophys. Res. Commun.* **120**, 885–890 (1984).
4. Masters, C. L. *et al. Proc. natn. Acad. Sci. U.S.A.* **82**, 4245–4249 (1985).

5. Selkoe, D. J., Abraham, C. R., Podlisny, M. B. & Duffy, L. K. *J. Neurochem.* **46**, 1820–1834 (1986).
6. Coria, F., Castano, E. M. & Frangione, B. *Am. J. Path.* **129**, 422–428 (1988).
7. Glenner, G. G. & Wong, C. W. *Biochem. biophys. Res. Commun.* **122**, 1131–1135 (1984).
8. Kang, J. *et al. Nature* **325**, 733–736 (1987).
9. Ponte, P. *et al. Nature* **331**, 525–527 (1988).
10. Tanzi, R. E. *et al. Nature* **331**, 528–530 (1988).
11. Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S. & Ito, H. *Nature* **331**, 530–532 (1988).
12. Selkoe, D. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 7341–7345 (1988).
13. Weidemann, A. *et al. Cell* **57**, 115–126 (1989).
14. St. George-Hyslop, P. H. *et al. Science* **238**, 664–666 (1987).
15. Goate, A. M. *et al. Lancet* **352**, 355 (1989).
16. Markesbery, W. R., Leung, P. K. & Butterfield, D. A. *J. Neurol. Sci.* **45**, 323–330 (1980).
17. Peterson, C., Gibson, G. E. & Blass, J. P. *N. Engl. J. Med.* **312**, 1063–1065 (1985).
18. Scudiero, D. A. *et al. Mutat. Res.* **159**, 125–131 (1986).
19. Peterson, C. & Goldman, J. E. *Proc. natn. Acad. Sci. U.S.A.* **83**, 2758–2762 (1986).
20. Zuberko, G. S., Wuslyko, M., Cohen, B. M., Boller, F. & Teplý, I. *Science* **238**, 539–542 (1987).
21. Tagliavani, F., Giaccone, G., Frangione, B. & Bugiani, O. *Neurosci. Lett.* **93**, 191–196 (1988).
22. Yamaguchi, H., Hirai, S., Morimatsu, M., Shoji, M. & Hangaya, Y. *Acta Neuropath.* **76**, 541–549 (1988).
23. Mann, D. & Esiri, M. M. *N. Engl. J. Med.* **318**, 789 (1988).
24. Joachim, C. L., Morris, J. & Selkoe, D. J. *Am. J. Path.* **135**, 309–319 (1989).
25. Glenner, G. G. in *Banbury Report: Biological Aspects of Alzheimer's Disease* (ed. Katzman, R.) 137–144 (Cold Spring Harbor Laboratory, New York, 1983).
26. Selkoe, D. J. *Neurobiol. Aging* **7**, 425–432 (1986).
27. Eikelenboom, P. & Stam, F. C. *Acta Neuropath.* **57**, 2139–2142 (1982).
28. Abraham, C. R., Selkoe, D. J. & Potter, H. *Cell* **52**, 487–501 (1988).
29. Coria, F. *et al. Lab. Invest.* **58**, 454–458 (1988).
30. Hart, M. N. *et al. Am. J. Path.* **132**, 167–172 (1988).
31. Joachim, C. L., Duffy, L. K., Morris, J. H. & Selkoe, D. J. *Brain Res.* **474**, 100–111 (1988).
32. Selkoe, D. J., Bell, D. S., Podlisny, M. B., Price, D. L. & Cork, L. C. *Science* **235**, 873–877 (1987).
33. Ihara, Y., Abraham, C. & Selkoe, D. J. *Nature* **304**, 727–730 (1983).
34. Dahl, D. & Bignami, A. *Brain Res.* **57**, 343–360 (1973).
35. Shirahama, T., Cohen, A. S. & Skinner, M. in *Advances in Immunohistochemistry* (ed. DeLellis, R. A.) 277–302 (Masson, New York, 1984).
36. Ju, S.-T., Skinner, M., Shirahama, T. & Cohen, A. S. *Fedn. Proc.* **46**, 1326 (1987).
37. Van Duinen, S. G. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 5991–5994 (1987).
38. Kitamoto, T., Ogomori, K., Tateishi, J. & Prusiner, S. B. *Lab. Invest.* **57**, 230–236 (1987).
39. Blöndal, H., Guomundsson, G., Benedikz, E. & Johannesson, G. *Alzheimer's Dis. Assoc. Dis.* **2**, 170 (1988).

ACKNOWLEDGEMENTS. We thank Marcia Podlisny, Dale Schenk and Lawrence Fritz for discussions. T. Shirahama, S.-T. Ju and M. Skinner provided the amyloid-AA antibodies. Supported by grants from the National Institute on Aging (LEAD Award) and Athena Neurosciences.

NMDA and non-NMDA receptors are co-localized at individual excitatory synapses in cultured rat hippocampus

John M. Bekkers & Charles F. Stevens

Section of Molecular Neurobiology, Howard Hughes Medical Institute, Yale University Medical School, 333 Cedar Street, New Haven, Connecticut 06510, USA

A CENTRAL assumption about long-term potentiation in the hippocampus is that the two classes of glutamate-receptor ion channel, the *N*-methyl-D-aspartate (NMDA) and the kainate/quisqualate (non-NMDA) subtypes, are co-localized at individual excitatory synapses^{1,2}. This assumption is important because of the perceived interplay between NMDA and non-NMDA receptors in the induction and expression of long-term potentiation: the NMDA class, by virtue of its voltage-dependent channel block by magnesium^{3,4} and calcium permeability^{5,6}, provides the trigger for the induction of long-term potentiation, whereas the actual enhancement of synaptic efficacy is thought to be provided by the non-NMDA class^{7,9}. If both receptor subtypes are present at the one synapse, such cross-modulation could occur rapidly and locally through diffusible factors. By measuring miniature synaptic currents in cultured hippocampal neurons we show that the majority (~70%) of the excitatory synapses on a postsynaptic cell possess both kinds of receptor, although to different extents. Of the remaining excitatory synapses, ~20% contain only the non-NMDA subtype and the rest possess only NMDA receptors. This finding provides direct evidence for co-localization of glutamate-receptor subtypes at individual synapses, and also points to the possibility that long-term potentiation might be differentially expressed at each synapse according to the mix of receptor subtypes at that synapse.

When synaptic transmission in cultured hippocampal

pyramidal neurons is blocked with tetrodotoxin (TTX), an ongoing spontaneous occurrence of small inward currents is observed (Fig. 1A, C; also ref. 10), which are similar to the spontaneous miniature endplate currents familiar in muscle fibres. These spontaneous events are thought to be due to the random release of quanta of neurotransmitter at individual excitatory synapses¹⁰. It is essential to our argument that this identification is correct, because we wish to infer from the properties of these miniature currents (which we have called 'minis') the distribution of glutamatergic receptors at single synaptic endings. In the study of these minis, we have examined four properties used to define neurotransmitter quanta: (1) small synaptic currents occur spontaneously when normal evoked synaptic transmission is blocked; (2) the minis have the same form and pharmacology as evoked currents; (3) the rate of occurrence is increased by the application of hypertonic solutions, even when normal release is blocked; and (4) the evoked synaptic currents arise by the summation of minis that occur with increased probability following stimulation.

First, in agreement with others¹⁰, we find that not only the addition of TTX, which blocks the sodium spike, but also the chelation of external calcium or the blocking of calcium channels with cobalt, which prevents transmitter release evoked by voltage, fails to abolish the spontaneous occurrence of minis. This parallels results obtained in other preparations¹¹, where it is well established that spontaneous miniature currents arise at individual synaptic endings. Second, the linear addition of a large number of minis yields a current that has the same form as an evoked excitatory postsynaptic current (e.p.s.c.) (Fig. 1). In Fig. 1A, *a–c*, three representative minis are shown with their rising edges aligned; the average of 58 such traces captured randomly from one cell is shown in Fig. 1A, *d*. This average is very similar in shape to the trace in Fig. 1B, which is a single record taken from another voltage-clamped cell in which an e.p.s.c. was elicited by firing an action potential in a nearby pyramidal neuron (note different vertical scale bar).

In addition to the fact that minis and e.p.s.c.s have the same form, components of the minis could, like the corresponding

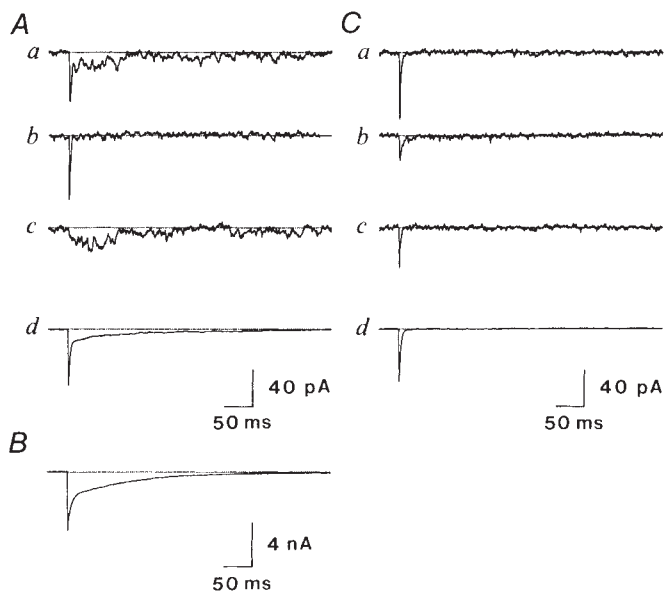


FIG. 1 Spontaneous minis sum to produce a current like an evoked e.p.s.c., and have a D-APV-sensitive slow component. All panels show the current recorded from a cultured hippocampal pyramidal cell voltage clamped at -60 mV. **A:** *a-c*, Representative spontaneous minis from one cell with their rising edges aligned; *d*, average of 58 minis, including those in *a-c*, from one cell. **B:** Excitatory postsynaptic current (e.p.s.c.) recorded in a cell in response to the firing of an action potential in another cell in the same culture dish. **C:** *a-c*, Spontaneous minis from the same cell as **A**, after bath perfusion of $100 \mu\text{M}$ D-APV; *d*, average of 32 minis, including those in *a-c*. **METHODS.** Hippocampal pyramidal cells were prepared from newborn rats as described⁶ and used after 7–18 days in culture. Currents were recorded by a patch electrode in the whole-cell mode; in panel **B** a second whole-cell electrode was used to fire the presynaptic cell. The bath solution normally contained (mM): NaCl (137), KCl (5), CaCl_2 (3), glucose (10), HEPES (5), pH 7.3, osmolality adjusted to 310 mOsm with sorbitol, to which was added $1 \mu\text{M}$ glycine, $1 \mu\text{M}$ strychnine, $100 \mu\text{M}$ picrotoxin and $1 \mu\text{M}$ TTX (no TTX in **B**). In some experiments the CaCl_2 was replaced by 2 mM CoCl_2 . External solution changes were effected by perfusing >10 times the chamber volume. The pipette solution contained (mM): CsCl (150), CsBAPTA (5) (1,2-bis(*o*-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid), HEPES (10), pH 7.2; in **B**, the presynaptic pipette contained the same solution, except that K^+ replaced Cs^+ so that the action potential repolarized rapidly. Experiments were at room temperature (23 – 25°C). Sources of chemicals: D-APV (Cambridge Research Biochemicals), CNQX (Tocris Neuramin), CsBAPTA and KBAPTA (Molecular Probes). Salts for bath solutions were from Alfa (Puratronic).

components of synaptic currents, be differentially blocked by specific glutamate-receptor antagonists. All of the recordings in Fig. 1 were made in a bath solution nominally without magnesium to optimize the detection of NMDA currents. The slow component in Fig. 1A, *d* and Fig. 1B, which by its kinetics indicates NMDA current¹², was abolished by $100 \mu\text{M}$ D-APV ($\text{D-2-amino-5-phosphonovaleric acid}$), a specific blocker of NMDA channels¹³. This is illustrated in Fig. 1C, in which representative individual traces (*a-c*) and the average of 32 such traces (*d*) are shown, taken from the same cell as in Fig. 1A after perfusion with D-APV. Bath perfusion with a high magnesium concentration instead of D-APV gave a similar result, whereas perfusion with $10 \mu\text{M}$ CNQX (6-cyano-2,3-dihydroxy-7-nitroquinoxaline), which blocks non-NMDA (K/Q) channels¹⁴, abolished the fast component of the spontaneous minis but did not affect the slow, noisy component (data not shown).

Third, we studied the effect of applying hypertonic solutions locally, which increases the frequency of spontaneous miniature endplate potentials (m.e.p.ps) at the neuromuscular junction by inducing asynchronous release of presynaptic vesicles¹⁵, even when normal transmitter release has been blocked. Results are

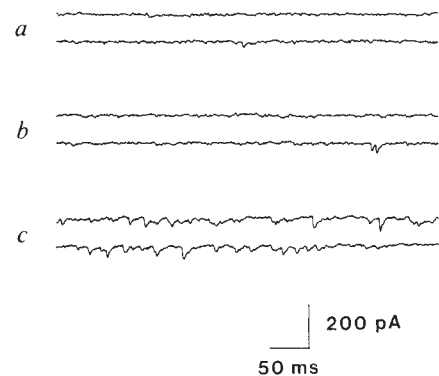
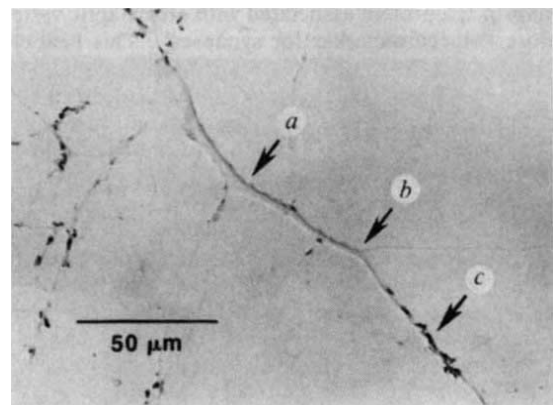


FIG. 2 Minis evoked by hypertonic solution are the same as spontaneous minis and can be evoked only where synapses are located. Top, a cultured pyramidal cell stained for synapsin I (dark granules) and showing three locations (*a, b, c*) where hypertonic solution was locally applied. Bottom, representative currents recorded while voltage clamping the soma at -60 mV and applying hypertonic solution at each of *a, b, c*. Altogether, 48 dendritic locations were studied in 9 different cells, for 43 of which the location of the hypertonic solution application could be unambiguously correlated with the results of the synapsin I stain. Of these, 30 locations contained stained boutons, and minis (excitatory and/or inhibitory) could be evoked at these locations by hypertonic solution. In none of the 13 locations without stained boutons could mini activity be evoked. The probability of a chance association between staining and activity for this number of trials is 2×10^{-5} (sign test).

METHODS. Electrophysiology and solutions as described in Fig. 1 legend, except that picrotoxin was omitted. Hypertonic solution (bath solution to which sucrose was added at 0.5 M) was applied in 300 ms bursts with a buffer pipette and removed by a nearby suction pipette (diameter $\sim 10 \mu\text{m}$) to restrict flow to a plume $\sim 15 \mu\text{m}$ wide. The cell was afterwards fixed in 4% formaldehyde and incubated overnight in synapsin I antibody diluted 1:100 in PBS; binding was localized with peroxidase.

shown in Fig. 2. Bath solution, made hypertonic by addition of sucrose, was applied locally using a puffer pipette at three points (*a, b, c*) on the dendrite of a pyramidal cell and the response recorded while voltage-clamping the soma. Application at point *c*, but not at *a* or *b*, resulted in a burst of small inward currents. These minis are evoked locally, $150 \mu\text{m}$ from the recording site on the cell body, a conclusion based on the observations that activity is produced by application of hypertonic solution only to certain locations, and that the mini currents are filtered by the dendritic cable to an extent which is dependent on the position at which the solution is applied¹⁶. The currents evoked by hypertonic solution are similar in size, kinetics, pharmacology and ionic selectivity to spontaneously occurring minis, similar to those illustrated in Fig. 1, although the particular currents shown in Fig. 2 appear different as a result of cable filtering.

The cell shown in Fig. 2 was then labelled using an antibody

to synapsin I, a protein associated with presynaptic vesicles and therefore a specific marker for synapses¹⁷. This histochemical marker revealed that the small inward currents could be evoked only at places where synapses are present (location *c*, Fig. 2). These results suggest that both spontaneous and hypertonic-solution-evoked minis arise from sporadic release of transmitter at individual synaptic endings.

Finally, we performed a quantal analysis to determine whether the statistical properties of the spontaneous minis were consistent with their being the basis of 'macroscopic' evoked synaptic currents. Examples of such currents are shown in Fig. 3 (insets); these are consecutive records in which an action potential was fired in a presynaptic cell and the postsynaptic response measured under voltage clamp. Each postsynaptic current was integrated to yield charge, which is a measure of the quantity of neurotransmitter released that is insensitive to jitter in the time at which individual quanta are released. Charge histograms were constructed from 62–455 consecutive records from the same cell pair in each of three different calcium concentrations (Fig. 3*b–d*). A charge histogram was also constructed for spontaneous minis recorded in the postsynaptic cell (Fig. 3*a*).

It is important to note the broadness of the distribution of mini sizes. In recordings from spontaneously occurring minis *in vivo*, we cannot tell how much of this size variation is an inherent property of the minis, and how much arises through cable distortion of synaptic currents produced at various locations over the dendritic tree. We have determined, however, that the spread in mini sizes obtained with sucrose applied locally at the soma, for which cable distortion is minimal, is the same as that shown in Fig. 3*a*. The wide range in mini size thus reflects inherent variability in individual events, at least in cultured neurons, and is not a consequence of cable attenuation.

The goal of our quantal analysis was to test whether the distribution of mini sizes (Fig. 3*a*), together with the assumption that the probability of occurrence of a single mini is binomially distributed, could predict the shapes of the evoked histograms (Fig. 3*b–d*). The fitting procedure was complicated by the fact that the distribution of mini sizes possessed a variance σ^2 that was significant compared with the variance, $\text{var}(q)$, of the

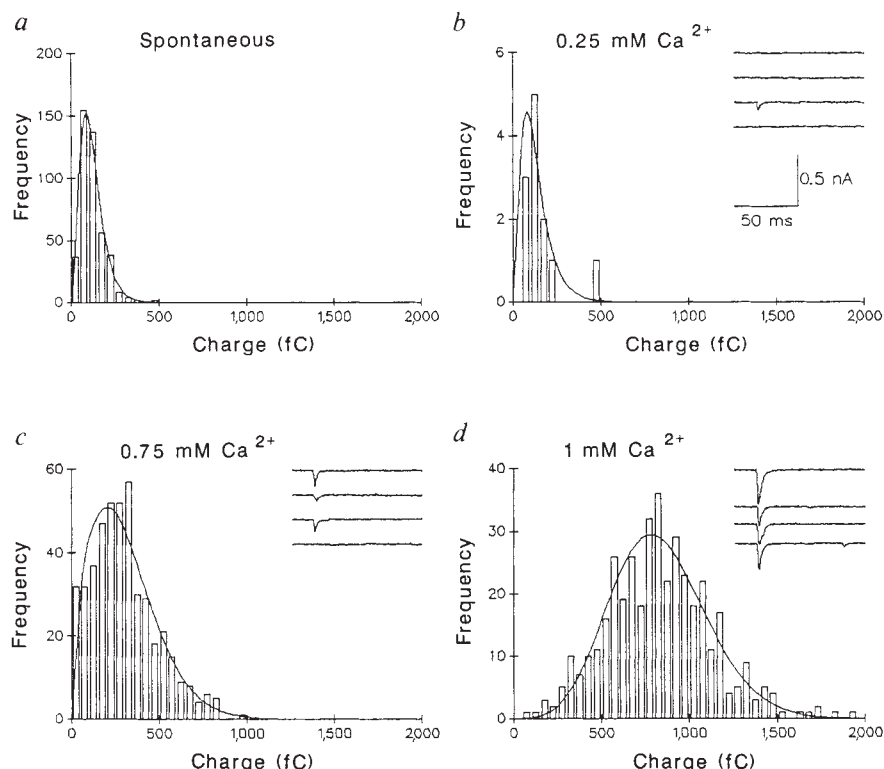
evoked distributions. Assuming independence and linear addition of the minis, it follows from elementary probability theory that $\bar{q} = \mu\bar{k}$, and $\text{var}(q) = \mu^2 s^2 + \bar{k}\sigma^2$, where $\bar{q}$ is the measured mean of the evoked distributions, μ is the measured mean of the spontaneous minis, $\bar{k} = Np$ and $s^2 = Np(1-p)$, where N and p are the usual binomial parameters. The two equations can thus be solved for the two unknowns N and p . This was done for each of the histograms in Fig. 3*b–d* (see figure legend for details) and the superimposed smooth curves are the calculated distributions. Note that the broadness of the underlying mini distribution acts to obliterate the discrete quantal peaks often observed in other preparations¹⁸. Excellent fits were also obtained for four other cell pairs in various calcium concentrations. These results show that evoked synaptic currents vary in amplitude in the way that would be expected if they were composed of the sum of probabilistically occurring minis.

Because our minis possess the four classes of characteristics that are used to define quantal events, we conclude that each mini in Fig. 1 reflects activation of receptors at a single excitatory synapse. Interestingly, some minis consisted entirely of slow, NMDA-type responses (Fig. 1*A, c*), some of fast, non-NMDA-type responses (Fig. 1*A, b*) and some of mixtures of both (Fig. 1*A, a*). This observation is quantified in Fig. 4, which shows superimposed histograms of the ratio slow-current/fast-current amplitude for mini e.p.s.cs recorded in the absence (bars) and presence (dotted line) of 100 μM D-APV. With D-APV the histogram is centred on the baseline—currents possess only the fast conductance—whereas without D-APV there is a distribution of mini e.p.s.cs with varying proportions of slow (NMDA) and fast (non-NMDA) currents. In this case ~110 out of 167 mini currents (66%) contained a mixture of NMDA and non-NMDA components and 16 (10%; far right bar) seemed to be pure NMDA currents, although it is probable that a number of these were missed owing to their small size and noisiness. Similar results were obtained in three other cells.

Assuming that the conductance of a single NMDA channel in these cells is the same at the synapse as at the soma (50 pS; ref. 6), at most ~8 NMDA-type channels are open in Fig. 1*A, a* and *c*. Single-channel fluctuations should be apparent in such

FIG. 3 Quantal analysis of the fast component of evoked e.p.s.cs from an isolated pair of pyramidal cells. The figure shows histograms of charge carried by spontaneous minis (*a*) and by evoked e.p.s.cs measured in the same postsynaptic cell in three different calcium concentrations (*b–d*). Insets show typical consecutive postsynaptic current traces, stimulated at 0.67 Hz; calibration bars in *b* apply to all insets.

METHODS. As in Fig. 1, except the bath omitted TTX and contained 10 mM Mg^{2+} , and both pre- and postsynaptic electrodes contained KCl. The postsynaptic cell was voltage-clamped at -60 mV, which is near the resting potential for these solutions. The smooth curve in *a* is a semi-empirical function (unpublished data) which was fitted by eye. Values for N and p were calculated as described in the text for each evoked histogram. The mean of these N values was found to be 14 ± 5 ($\pm$ s.d., $n=3$) and N was constrained to this mean for all calcium concentrations. The predicted distribution was then calculated using a convolution technique that takes into account the mini distribution in panel *a*, and the value of p was adjusted slightly for each panel until a good visual fit was obtained. The smooth curves in *b–d* show predicted distributions for the following values of p : 0.02 (0.25 Ca^{2+}), 0.18 (0.75 Ca^{2+}), 0.56 (1 Ca^{2+}). The observed and predicted numbers of failures were, respectively: 50 and 47 (0.25 Ca^{2+}), 24 and 28 (0.75 Ca^{2+}), 0 and 0 (1 Ca^{2+}).



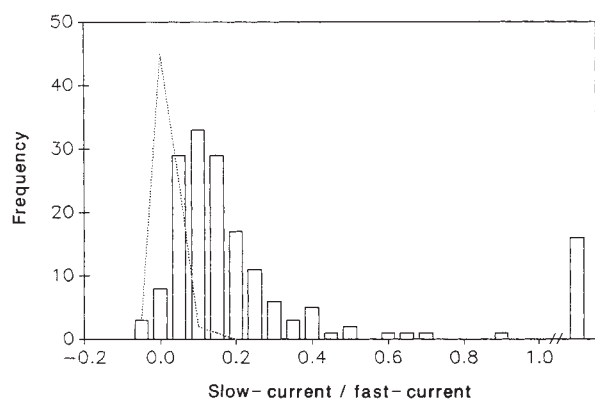


FIG. 4 Histograms showing the distribution of size of the slow, D-APV-sensitive current relative to the size of the fast current in spontaneous mini e.p.s.cs. Bars (167 events measured), without D-APV; dotted line (76 events measured), with D-APV; all taken from the same cell as shown in Fig. 1A and C. The fast amplitude was measured at the peak of the spike occurring 1–2 ms after the foot of the mini, at which time the NMDA current has only just begun to turn on (unpublished data); the slow amplitude was measured by averaging over a 10 ms window starting 10 ms after the peak of the fast spike. The bar to the far right represents 16 minis that seemed to consist purely of slow component.

a small population, and this accords with the noisy appearance of the traces. With a conductance for the non-NMDA channels of 10 pS (ref. 6), the fast component of the minis in Fig. 1A, *a* and *b*, would require ~110 such channels to be open at the peak, although a recent suggestion¹⁹ that the fast component arises from channels with a 35 pS conductance would imply that only about 30 channels are open. The above channel numbers might be up to twice as large at some synapses, judging by the spread in mini amplitudes from synapse to synapse.

Typically, about 70% of the mini e.p.s.cs we measured possessed both fast and slow components. Assuming that the probability of occurrence of a mini was the same at all synapses, irrespective of the mix of receptor subtypes at that synapse, about 30% of the excitatory synapses on our cells contained only NMDA or only non-NMDA-type receptor-channels. It is not known if a similar situation arises in hippocampal slices, where long-term potentiation has been most actively studied. It is interesting to consider, however, whether long-term potentiation could be induced at a synapse that does not contain both subtypes. This would require the study of long-term potentiation at only a few synapses, rather than by the usual means of averaging the contributions of many synapses.

In addition to showing that NMDA and non-NMDA channel subtypes are co-localized at individual synapses, we have also found that the statistical model for quantal release first developed for the neuromuscular junction²⁰ also seems adequate for excitatory synapses in the hippocampus. The analysis is complicated, however, by the fact that individual quanta vary considerably in size. We anticipate that this description of transmitter release will be useful in distinguishing between pre- and postsynaptic mechanisms of long-term potentiation. □

13. Watkins, J. C. & Evans, R. H. A. *Rev. Pharmac. Tox.* **21**, 165–204 (1981).
14. Honore, T. *et al. Science* **241**, 701–703 (1988).
15. Fatt, P. & Katz, B. *J. Physiol., Lond.* **117**, 109–128 (1952).
16. Bekkers, J. M. & Stevens, C. F. *Understanding the Brain through the Hippocampus: The Hippocampal Region as a Model for Studying Brain Structure and Function* (eds Storm-Mathisen, J., Zimmer, J. & Ottersen, O. P.) (Elsevier, Amsterdam, in the press).
17. De Camilli, P., Cameron, R., Greengard, P. *J. Cell Biol.* **96**, 1337–1354 (1983).
18. Martin, A. R. *Handbook of Physiology. The Nervous System* 329–355 (Am. Physiol. Soc. **1**, 1977).
19. Tang, C.-M., Dichter, M. & Morad, M. *Science* **243**, 1474–1477 (1989).
20. del Castillo, J. & Katz, B. *J. Physiol.* **124**, 560–573 (1954).

ACKNOWLEDGEMENTS. We thank Pietro De Camilli for the synapsin I antibody and Gary Banker for help with immunocytochemistry. Supported by the Howard Hughes Medical Institute (C.F.S.).

Glucose-inhibition of glucagon secretion involves activation of GABA_A-receptor chloride channels

Patrik Rorsman, Per-Olof Berggren*, Krister Bokvist, Hans Ericson†, Hanns Möhler‡, Claes-Göran Östenson* & Paul A. Smith§

Department of Medical Physics, Gothenburg University, Box 33031, S-400 33 Gothenburg, Sweden

* Department of Endocrinology, Karolinska Institute, Karolinska Hospital, Box 60500, S-104 01 Stockholm, Sweden

† Department of Anatomy, Biomedicum, Box 571, S-751 23 Uppsala, Sweden

‡ Pharmaceutical Research Department, F. Hoffmann-La Roche & Co. Ltd., CH-4002 Basel, Switzerland

THE endocrine part of the pancreas plays a central role in blood-glucose regulation. It is well established that an elevation of glucose concentration reduces secretion of the hyperglycaemia-associated hormone glucagon from pancreatic α_2 cells. The mechanisms involved, however, remain unknown. Electrophysiological studies have demonstrated that α_2 cells generate Ca^{2+} -dependent action potentials. The frequency of these action potentials, which increases under conditions that stimulate glucagon release, is not affected by glucose or insulin¹. The inhibitory neurotransmitter γ -aminobutyric acid (GABA) is present in the endocrine part of the pancreas at concentrations comparable to those encountered in the central nervous system², and co-localizes with insulin in pancreatic β cells³. We now describe a mechanism whereby GABA, co-secreted with insulin from β cells, may mediate part of the inhibitory action of glucose on glucagon secretion by activating GABA_A-receptor Cl^- channels in α_2 cells. These observations provide a model for feedback regulation of glucagon release, which may be of significance for the understanding of the hypersecretion of glucagon frequently associated with diabetes⁴.

Immunostaining of pancreatic islet cells for GABA_A-receptor revealed a positive reaction in glucagon-containing α_2 cells and somatostatin-containing α_1 cells (Fig. 1*a, d*), but not in β cells. The α_1 cells were generally smaller than the α_2 cells and were characterized by an irregular morphology with projecting cellular processes. Such cells were excluded from electrophysiological recordings in favour of round cells with large diameters corresponding to α_2 cells (20–25 μm ; cell capacitance 10–20 pF). The existence of GABA_A receptors on α_1 cells is not surprising in view of its reported inhibitory effect on somatostatin secretion⁵.

The top trace of Fig. 1*e* shows a membrane-potential recording from a single α_2 cell. The cell generates spontaneous overshooting Ca^{2+} -dependent action potentials from –60 mV. Application of 100 μM GABA results in a hyperpolarization of ~15 mV and the cessation of spiking activity. Withdrawal of GABA leads to depolarization and the reappearance of action

Received 2 May; accepted 11 August 1989.

1. Nicoll, R. A., Kauer, J. A. & Malenka, R. C. *Neuron* **1**, 97–103 (1988).
2. Brown, T. H., Chapman, P. F., Kairiss, E. W. & Keenan, C. L. *Science* **242**, 724–728 (1988).
3. Nowak, L., Bregestovski, P., Ascher, P., Herbet, A. & Prochiantz, A. *Nature* **307**, 462–465 (1984).
4. Mayer, M. L., Westbrook, G. L. & Guthrie, P. B. *Nature* **309**, 261–263 (1984).
5. MacDermott, A. B., Mayer, M. L., Westbrook, G. L., Smith, S. J. & Barker, J. L. *Nature* **321**, 519–522 (1986).
6. Jahr, C. E. & Stevens, C. F. *Nature* **325**, 522–525 (1987).
7. Kauer, J. A., Malenka, R. C. & Nicoll, R. A. *Neuron* **1**, 911–917 (1988).
8. Müller, D., Joly, M. & Lynch, G. *Science* **242**, 1694–1697 (1988).
9. Davies, S. N., Lester, R. A. J., Reymann, K. G. & Collingridge, G. L. *Nature* **338**, 500–503 (1989).
10. Finch, D. M. & Jackson, M. B. *Neurosci. Abstr.* **13**, 310 (1987).
11. Miledi, R. & Thies, R. *J. Physiol., Lond.* **212**, 245–257 (1971).
12. Forsythe, I. D. & Westbrook, G. L. *J. Physiol., Lond.* **396**, 515–533 (1988).

§ Present address: University Laboratory of Physiology, Parks Road, Oxford OX1 3PT, UK

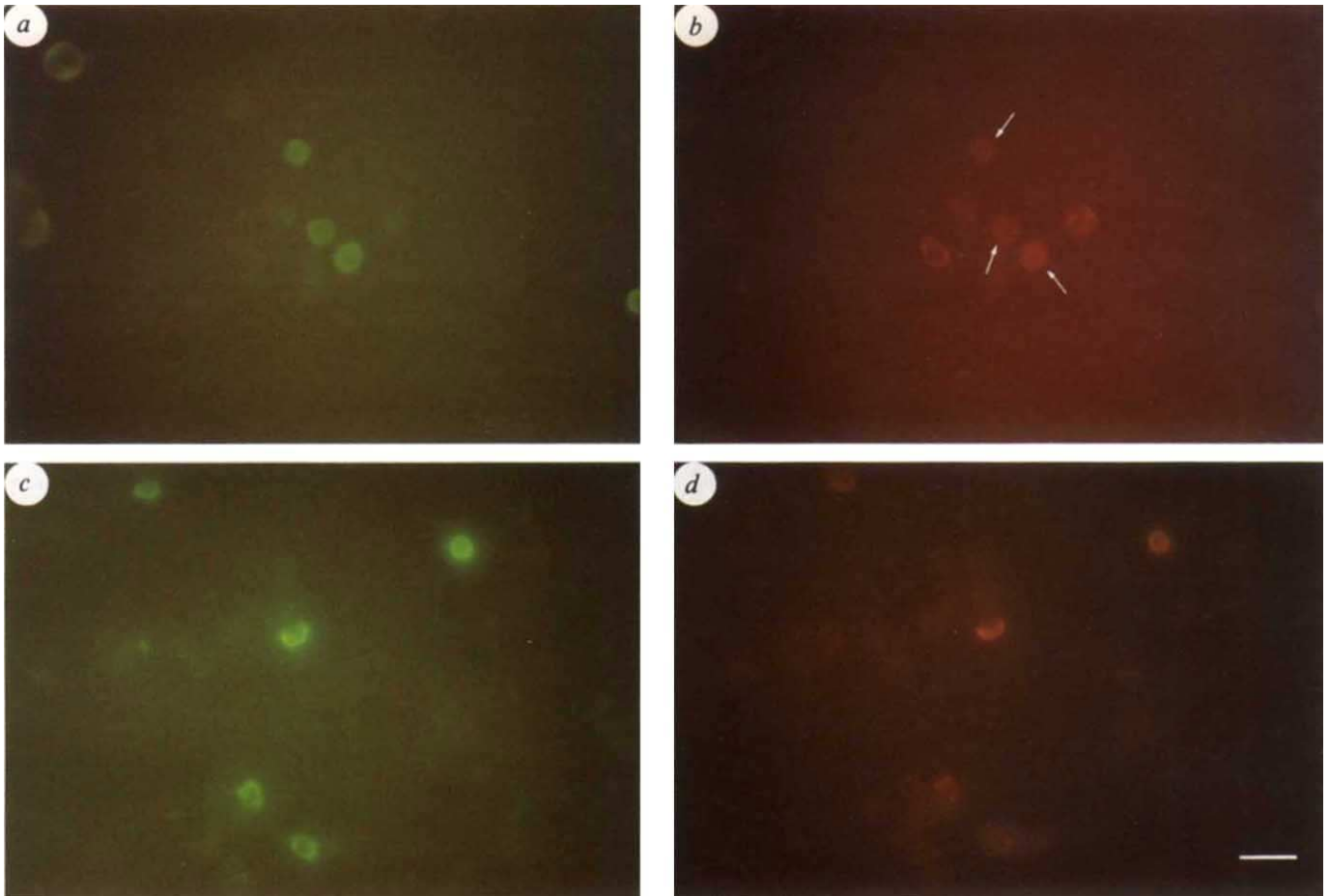
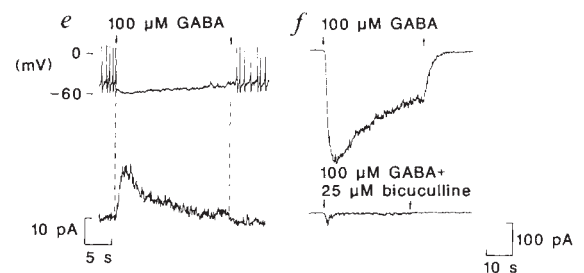


FIG. 1 *a*, Photomicrograph illustrating glucagon immunoreactive guinea-pig pancreatic islet α_2 cells. *b*, Photomicrograph demonstrating that a number of the glucagon immunoreactive cells in *a*, were also immunoreactive against GABA_A-receptor antibodies (arrows). Somatostatin immunoreactive guinea-pig pancreatic α_1 cells (*c*) were also immunoreactive against GABA_A-receptor antibodies (*d*). Bar, 25 μ m. *e*, Top, membrane potential recording from a guinea-pig α_2 cell. Application of 100 μ M GABA (indicated by the arrows and the vertical dashed lines) results in a hyperpolarization and the cessation of spiking activity; bottom, whole-cell voltage-clamp current record from same cell. Cell was clamped at -40 mV. Outward current was activated by GABA. Pipette was filled with solution containing low $[Cl^-]_i$. *f*, Top, current response evoked by GABA. With the solutions used, $[Cl^-]$ is about the same on both sides of the membrane. At negative membrane potentials, chloride ions will therefore leave the cell, giving rise to an inward current. With low $[Cl^-]_i$, currents will be outward as in *e*. Bottom, suppression of response to 100 μ M GABA after inclusion of 25 μ M bicuculline (dissolved in dimethyl sulphoxide; final concentration 0.1%).

METHODS. Guinea-pig pancreatic islet cells were prepared and cultured for 1–3 days as previously described¹. The cells, which attach spontaneously to the bottom of the tissue-culture dishes, were fixed for ~5 min with PLP-fixative¹⁶ containing 0.01 M periodate, 0.075 M D,L-lysine and 2% paraformaldehyde in 0.1 M phosphate buffer. For storage, the fixative was removed and replaced with normal extracellular solution. The cells were pretreated with 0.2% Triton X-100 for 1 h and then incubated for 3 days in rabbit anti-glucagon (Novo, Sweden), rabbit anti-somatostatin (Bio-Bol, Sweden) antibodies diluted 1:3,000 and 1:1,000, respectively, or in 100% mouse hybridoma (bd-17) supernatant—an antibody against the β -subunit of the GABA_A receptor. The preparation and characteristics of the antibody have previously been described^{17,18}. The β -subunit protein, recognized by bd-17, is part of the GABA_A-receptor Cl^- -channel complex. When coexpressed with the α -subunit protein in *Xenopus* oocytes, GABA-gated chloride channels are formed¹⁹. To visualize the antigen-antibody complexes, the cells were incubated in fluorescein-isothiocyanate labelled porcine anti-rabbit antibodies (Dakopatts, Copenhagen) diluted 1:100 in PBS medium followed by an incubation for 30 min in the immunoglobulin fraction of rabbit normal serum (Dakopatts) diluted 1:10 in PBS. Finally, the cells were incubated for



1 h in rhodamine-conjugated rabbit anti-mouse antibodies (Dakopatts) diluted 1:100. The whole-cell configuration of the patch-clamp technique²⁰ was used to record membrane potentials and currents. Pancreatic islets were isolated from guinea-pigs by collagenase digestion. The isolated islets were dissociated into single cells using trypsin, plated on coverslips and maintained in tissue-culture (RPMI 1640 supplemented with 10% v/v FCS, with antibiotics) for up to one week. The α_2 cells were identified on the basis of their large size (diameter 20–30 μ m; cell capacitance >10 pF) as previously described¹. Immunohistochemistry confirmed that the majority of large cells were glucagon-containing α_2 cells. Throughout the electrophysiological experiments, the cells were immersed in an extracellular solution containing (in mM): NaCl, 138; KCl, 5.6; $MgCl_2$, 1.2; $CaCl_2$, 2.6; HEPES-NaOH (pH 7.4), 5. The pipette-filling solution in the membrane-potential recording experiment *e* was composed of (in mM): K-glutamate, 125; $MgCl_2$, 1; EGTA, 10; MgATP, 3; HEPES-HCl, 5 (pH 7.15); total $[Cl^-]_i = 4.5$ mM. In *f*, the pipette was filled with a medium containing (in mM): N-methyl-D-glucamine (NMG), 150; HCl, 110; EGTA, 10; $MgCl_2$, 1; MgATP, 3; HEPES-HCl, 5 (pH 7.15); total $[Cl^-]_i = 120$ mM. An EPC7 (List Electronic, Darmstadt, FRG) patch-clamp amplifier was used for the electrophysiological recordings. Pipettes were pulled from borosilicate or aluminosilicate glass (Hilgenberg, Malsfeld, FRG) and had resistances of 4–10 M Ω . The current and voltage signals were recorded on magnetic tape using a Racal 4 Store FM tape recorder.

potentials. Recordings of spiking activity from cell-attached patches on arginine-stimulated cells revealed a similar inhibition by GABA (data not shown). The bottom trace shows a whole-cell voltage-clamp current record from the same cell. It was observed that the inhibition of the electrical activity is coincident with the activation of an outward current. In Fig. 1*f*, we show that the current elicited by GABA is completely and reversibly blocked by 25 μM of the GABA_A-receptor antagonist bicuculline. Similar effects were obtained with 25 μM picrotoxin (data not shown). The currents elicited by GABA depend on the intracellular Cl^- concentration, as shown in Fig. 2*a*. The reversal potential was shifted from $-8 \pm 2 \text{ mV}$ ($n=3$) when recorded with an intracellular Cl^- -concentration ($[\text{Cl}^-]_i$) of 120 mM, to $-45 \pm 5 \text{ mV}$ ($n=3$), when recorded with a $[\text{Cl}^-]_i$ of 20 mM. This shift of 37 mV is in reasonable agreement with that expected from the Nernst equation for a sixfold change of $[\text{Cl}^-]_i$. Figure 2*b* shows the single-channel events that produce the whole-cell currents evoked by GABA, panel (i). The current voltage (i - V) relationships recorded with low and high $[\text{Cl}^-]_i$ for the most common conductance state are shown in panel (ii). The single-channel conductances were $29 \pm 2 \text{ pS}$ ($n=6$) for the inward current and $18 \pm 1 \text{ pS}$ ($n=6$) for the outward currents when recorded with high $[\text{Cl}^-]_i$ (120 mM). The latter value corresponds to the $17 \pm 1 \text{ pS}$ ($n=4$) obtained when recording outward currents with low $[\text{Cl}^-]_i$. The single-channel currents could be fitted to the constant field current equation in the voltage range -80 to $+60 \text{ mV}$. Assuming an activity constant of 0.75 for Cl^- (ref. 6), we obtained estimates of P_{Cl} of $(6.2 \pm 0.2) \times 10^{-13} \text{ cm}^3 \text{ s}^{-1}$ ($n=5$) and $(6.0 \pm 0.1) \times 10^{-13} \text{ cm}^3 \text{ s}^{-1}$ ($n=4$) for high and low $[\text{Cl}^-]_i$, respectively. From the observations in Figs 1 and 2 it can be concluded that the currents elicited by GABA in the α_2 cells are carried by GABA_A-receptor Cl^- channels similar to those described in other cell types⁶⁻⁹.

The concentration dependence of GABA-evoked currents is shown in Figure 3*a*. The current amplitude was extremely variable and, for a pulse of 100 μM GABA, ranged between a few to several hundred picoamperes in cells of a similar size. Figure 3*b* shows a dose-response curve. At concentrations $\geq 30 \mu\text{M}$, currents desensitize and true peak current amplitudes were

estimated by extrapolation of the decaying part of the current response to time zero. The sigmoidal curve was obtained by approximating the data to the equation $I(C)/I_{\text{max}} = C^n/(C^n + K_a^n)$ (equation 1), where $I(C)$ is the Cl^- current elicited by a GABA concentration of C , I_{max} the maximal current amplitude, n the Hill factor and K_a the concentration of GABA eliciting a half-maximal current response. In six experiments, average values of K_a and n were $40 \pm 6 \mu\text{M}$ and 2.0 ± 0.3 , respectively. The latter value is similar to that reported in other cells^{7,10,11} and suggests that the binding of two molecules of GABA is required for a channel opening in the α_2 cell. The currents evoked by 30 μM GABA were approximately doubled in the presence of 10 μM of the benzodiazepine diazepam, or after replacement of GABA by muscimol (data not shown), as expected for GABA_A receptor Cl^- currents.

The effects of GABA and bicuculline on glucagon secretion from isolated islets is shown in Table 1. Arginine enhanced glucagon secretion 1.6-fold. Addition of 100 μM GABA counteracted this effect and reduced the arginine-stimulated release by 70%. This effect of GABA was abolished by addition of 100 μM bicuculline. Inhibition of glucagon secretion was also observed when glucose was increased to 16.7 mM in the absence of arginine. This effect was also counteracted (down 50%) by bicuculline. This suggests that GABA_A-receptor Cl^- -channels are indeed involved in glucose regulation of glucagon release and that the interstitial GABA concentration increases to physiologically active levels during stimulation of insulin secretion. We acknowledge that although we are able to detect GABA being released from the pancreatic islets, we have so far failed to demonstrate an enhancement by glucose. It should be emphasized, however, that glucose has been reported to increase not only the release of insulin, but also that of GABA¹². The failure of bicuculline to completely suppress the inhibitory effect of supraphysiological glucose concentrations suggests that part of this inhibition results from a mechanism not involving GABA_A receptor activation^{13,14}. Somatostatin is a potent inhibitor of glucagon release. As α_1 cells are equipped with GABA_A receptors, it is important to exclude that the action of GABA on glucagon release is secondary to a change in the

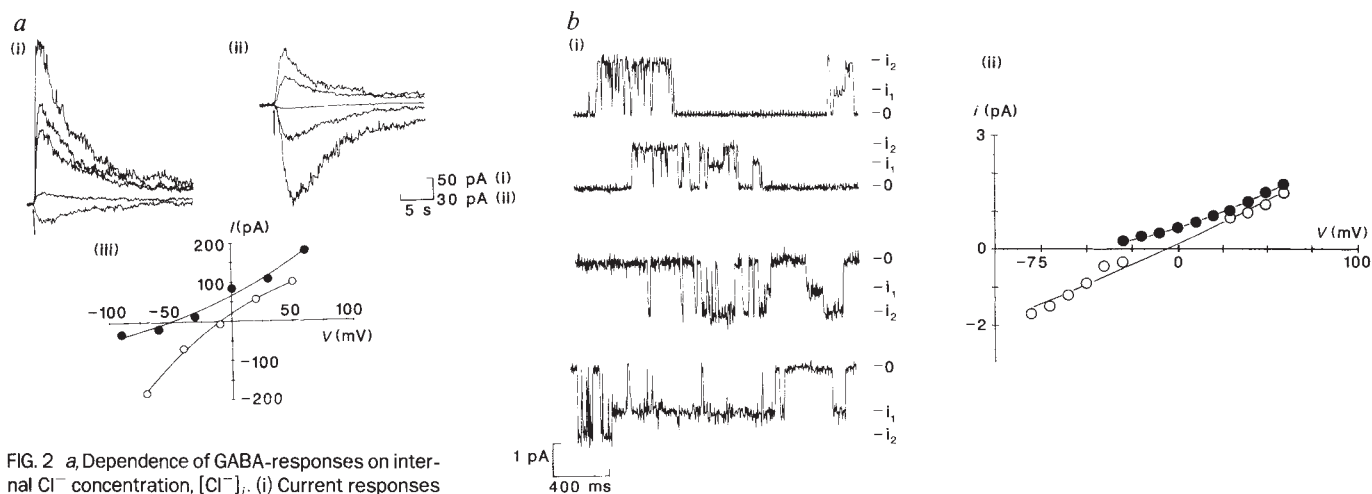
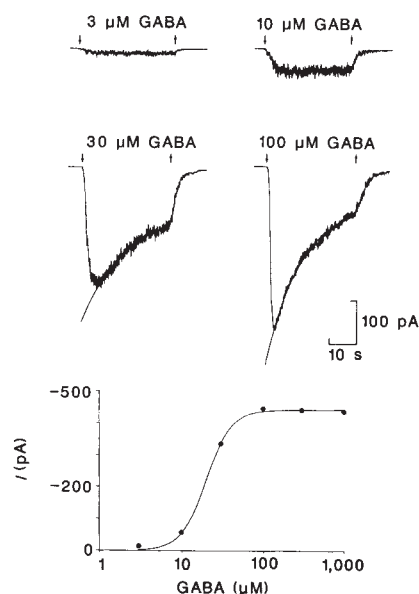


FIG. 2 *a*, Dependence of GABA-responses on internal Cl^- concentration, $[\text{Cl}^-]_i$. (i) Current responses elicited by 100 μM GABA at different membrane potentials using a pipette filled with low $[\text{Cl}^-]_i$. (ii) Current responses elicited by 100 μM GABA at different membrane potentials using a pipette with high $[\text{Cl}^-]_i$. (iii) Whole cell current (I)-voltage (V) relationship for the peak currents in (i) and (ii). The curves were drawn according to the function $I(V) = A(V - V_r) \exp [B(V - V_r)]$ where $I(V)$ is the peak current at a voltage V and V_r is the reversal potential. The values of V_r , A and B were derived from an iterative least-squares routine. *b*, (i) Single-channel activity evoked by 10 μM GABA recorded from an outside-out patch at membrane potentials (from top to bottom) of +70, +50, -50 and -70 mV recorded with high $[\text{Cl}^-]_i$. At least two conductance levels are seen (marked i_1 and i_2). (ii) Single

GABA-receptor channel i - V relationships of the most common conductance state. Currents were recorded with $[\text{Cl}^-]_i$ of 20 mM (solid circle) or 120 mM (open circle). The solid lines represent fits to the constant field current equation where P_{Cl} equals $6 \times 10^{-13} \text{ cm}^3 \text{ s}^{-1}$ ($[\text{Cl}^-]_i = 20 \text{ mM}$; solid circle) and $5.7 \times 10^{-13} \text{ cm}^3 \text{ s}^{-1}$ ($[\text{Cl}^-]_i = 120 \text{ mM}$; open circle).

METHODS. The high- $[\text{Cl}^-]_i$ -solution was the NMG-medium specified in the legend to Fig. 1. The low- $[\text{Cl}^-]_i$ -solution contained (in mM): NaCl, 14; Na-glutamate, 117; MgCl_2 , 1; EGTA, 10; MgATP , 3; HEPES-HCl, 5 (pH 7.15); $[\text{Cl}^-]_i \sim 20 \text{ mM}$. Single-channel currents were filtered at 0.2 kHz (-3 dB) using a 4-pole Bessel filter.

FIG. 3 Dependence on GABA concentration of Cl^- current responses. *a*, 3, 10, 30 and 100 μM GABA was applied to a single α_2 cell at intervals >1 min as indicated by the upward and downward arrows. It was ascertained that the periods between the respective exposures were sufficient to permit recovery from desensitization. Peak amplitudes were obtained by either direct measurement (3 and 10 μM GABA) or extrapolation to zero time by fitting exponential functions (indicated by the superimposed lines) to the decaying part of the currents (30 and 100 μM GABA). Currents were recorded using NMG-filled pipettes (see Fig. 1 legend). Cell was held at -70 mV. *b*, Dose-response curve. The straight line was obtained by fitting the peak currents observed in the presence of 3, 10, 30, 100, 300 and 1,000 μM GABA to equation 1 (see text). The currents have been corrected for desensitization at concentrations ≥ 30 μM .



secretion of somatostatin. Somatostatin secretion under basal conditions averaged 1.2 ± 0.3 pg per 100 islets min^{-1} ($n = 3$) and increased to 11.8 ± 1.4 pg per 100 islets min^{-1} upon stimulation by 10 mM arginine ($P < 0.001$). The addition of 100 μM GABA did not measurably affect somatostatin secretion, but inclusion of 100 μM bicuculline resulted in an increase to 19.9 ± 2.0 pg per 100 islets min^{-1} ($P < 0.01$). The latter effect suggests that the interstitial concentration of GABA within the arginine-stimulated islet is sufficient to produce a substantial activation of GABA_A -receptor Cl^- -channels in the α_1 cell. Somatostatin levels were measured using radioimmunoassay¹⁵. From these observations it seems unlikely that changes in the secretion of somatostatin mediate the influence of GABA on glucagon release. In fact, the stimulation of somatostatin secretion obtained after addition of bicuculline would tend to counteract

the stimulatory actions of the latter on glucagon release. This may be the reason why GABA_A receptor antagonists fail to completely reverse the glucose inhibition.

We propose that glucose inhibition of glucagon secretion involves the activation of GABA_A -receptor Cl^- -channels by GABA co-secreted with insulin from β -cells. This hyperpolarizes the α_2 cell, inhibits spiking, lowers cytoplasmic free Ca^{2+} and reduces glucagon secretion. This model emphasizes the role of paracrine regulatory pathways made possible by the complicated architecture of the endocrine pancreas and provides a flexible system for regulation of glucagon secretion. Any situations associated with the stimulation of insulin secretion will consequently lead to suppression of glucagon release. The model predicts that glucagon secretion will increase under conditions of impaired β -cell function. In fact, non-insulin-dependent diabetes mellitus is often associated with hypersecretion of glucagon, resulting in an aggravation of the diabetogenic action of hypoinsulinaemia⁴. □

TABLE 1 Effects of GABA on glucagon secretion

Line	Incubation medium additive	Glucagon secretion (pg per islet per 30 min)	n
1	1.7 mM glucose	11.8 ± 1.6	8
2	1.7 mM glucose + 10 mM L-arginine	$19.2 \pm 1.8^*$	8
3	1.7 mM glucose + 10 mM L-arginine + 100 μM GABA	$13.9 \pm 1.0^\dagger$	7
4	1.7 mM glucose + 10 mM L-arginine + 100 μM GABA + 100 μM bicuculline	$18.2 \pm 1.3^\ddagger$	5
5	16.7 mM glucose	$7.5 \pm 0.5^\S$	8
6	16.7 mM glucose + 100 μM bicuculline	$9.6 \pm 0.7\parallel$	5

Pancreatic islets were isolated by collagenase digestion from male guinea-pigs weighing 200–250 g. After tissue culture overnight, batches of 10 islets were incubated in Krebs-Ringer bicarbonate buffer solution (pH 7.4) supplemented with 10 mM HEPES, 2 mg ml^{-1} BSA (fraction V), and additions as in the Table. Incubations were performed for 30 min at 37 °C in a gas phase of 5% CO_2 :95% O_2 . After incubation, aliquots of the medium were immediately removed for glucagon radioimmunoassay using the 30K antibody²¹. Values are means $\pm$ s.e.m. of indicated number of experiments, each experiment consisting of duplicate or triplicate incubations with islets from one animal. Significances of differences were evaluated using Student's *t*-test. * $P < 0.01$ versus line 1; $^\dagger P < 0.05$ versus line 2; $^\ddagger P < 0.05$ versus line 3; $^\S P < 0.05$ versus line 1; $\parallel P < 0.05$ versus line 5. Difference between lines 1 and 6 not statistically significant ($P > 0.1$).

Received 5 May; accepted 8 August 1989.

- Rorsman, P. & Hellman, B. *J. gen. Physiol.* **91**, 223–242 (1988).
- Okada, Y., Taniguchi, H. & Shimada, C. *Science* **194**, 620–622 (1979).
- Garry, D. J., Sorenson, L., Elde, R. P., Maley, B. E. & Madsen, A. *Diabetes* **35**, 1090–1096 (1986).
- Lins, P. E., Wajngot, A., Adamsson, U., Vranic, M. & Efendic, S. *Diabetes* **32**, 633–635 (1983).
- Robbins, M. S., Grouse, L. H., Sorenson, R. L. & Elde, R. P. *Diabetes* **30**, 168–171 (1981).
- Bormann, J., Hamill, O. P. & Sakmann, B. *J. Physiol. (Lond)* **385**, 243–286 (1987).
- Bormann, J. & Clapham, D. E. *Proc. natn. Acad. Sci. U.S.A.* **82**, 2168–2172 (1985).
- Vicini, S., Mienville, J. M. & Costa, E. *J. Pharmac. exp. Therap.* **243**, 1195–1201 (1987).
- Taleb, O. et al. *Pflügers Arch. ges. Physiol.* **409**, 620–631 (1987).
- Kehl, S. J., Hughes, D. & McBurney, R. N. *Br. J. Pharmac.* **92**, 573–585 (1987).
- Akaike, N., Inoue, M. & Krishtal, O. A. *J. Physiol. (Lond)* **379**, 171–185 (1986).
- Gerber, J. C. & Hare, T. A. *Brain Res. Bull.* **5**, (suppl. 2), 341–346 (1980).
- Johansson, H., Gylfe, E. & Hellman, B. *Biochem. biophys. Res. Commun.* **147**, 309–313 (1987).
- Pipeleers, D. G., Schuit, F. C., Van Schravendijk, C. F. H. & Van De Winkel, M. *Endocrinology* **117**, 817–823 (1985).
- Falona, G. R. & Unger, R. H. In *Methods of Hormone Radioimmunoassay* (eds Jaffe B. M. & Behrman H. R.) 324–326 (Academic, New York, 1974).
- McLean, I. W. & Nakane, P. K. *J. Histochem. Cytochem.* **22**, 1077–1083 (1974).
- Sooch, P. et al. *Nature* **314**, 168–171 (1985).
- Häring, P. et al. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4837–4841 (1985).
- Schofield, P. R. et al. *Nature* **328**, 221–227 (1987).
- Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers Arch. ges. Physiol.* **391**, 85–100 (1981).
- Efendic, S., Nylen, A., Roovete, A. & Uvnäs-Wallensten, K. *FEBS Lett.* **92**, 33–35 (1978).

ACKNOWLEDGEMENTS. We thank Dr W. T. O'Connor for GABA measurements and Dr F. M. Ashcroft for discussion and help with manuscript preparation. This work was supported by Å. Wibergs Stiftelse, Syskonen Svenssons Fond för Medicinsk Forskning, M. Bergvalls Stiftelse, Amundsons Fond, O.E. och Edla Johanssons Fond, Svenska Hoechst Diabetesfond, Nordisk Insulinfond, Svenska Diabetesförbundet, the Swedish Medical Research Council, Svenska Läkaresällskapet, the Bank of Sweden Tercentenary Foundation, Novo Industries, Clas Groschinskys Minnesfond, Familjen Ernors Fond, Tore Nilsons Fond för medicinsk forskning, the Aage and Louis Hansens Memorial Foundation, Stiftelsen L. Hiertas Minne, W. och M. Lundgrens vetenskapsfond, Svenska Sällskapet för Medicinsk Forskning, funds at the Karolinska Institute and the Medical Faculty, Gothenburg University. P.R. is a recipient of a postdoctoral fellowship from the Swedish Medical Research Council and P.S. was funded by a Royal Society European Exchange Fellowship.

A neuromodulator of synaptic transmission acts on the secretory apparatus as well as on ion channels

Helen Man-Son-Hing, Mark J. Zoran, Ken Lukowiak* & Philip G. Haydon†

Department of Zoology, Iowa State University, Ames, Iowa 50011, USA
* Neuroscience Research Group, University of Calgary, Calgary, Alberta, Canada T2N 4N1

THE mechanisms that underlie synaptic plasticity have been largely inferred from electrophysiological studies performed at sites remote from synaptic terminals. Thus the mechanisms involved in plasticity at the secretory sites have remained ill-defined. We have now used somatic synapses of cultured *Helisoma* neurones¹⁻³ to directly assess presynaptic ion conductances and study the secretory apparatus. At these synapses we determined the actions of a modulatory neuropeptide, Phe-Met-Arg-Phe-NH₂ (FMRFa), on the release of the neurotransmitter acetylcholine (ACh). Using voltage- and calcium-clamp techniques, we have demonstrated that FMRFa causes a presynaptic inhibition of ACh release by (1) reducing the magnitude of the voltage-dependent calcium current, and (2) regulating the secretory apparatus. The photolabile calcium cage, nitr-5 (refs 3-8), was dialysed into the presynaptic cell. In response to ultraviolet light, calcium was released from nitr-5 and

ACh secretion was stimulated. Under conditions of constant internal calcium, FMRFa reduced the rate of ACh release. Thus we conclude that FMRFa reduces the influx of calcium during the action potential and decreases the sensitivity of the secretory apparatus to elevated internal calcium, thereby contributing to a presynaptic inhibition of transmitter release.

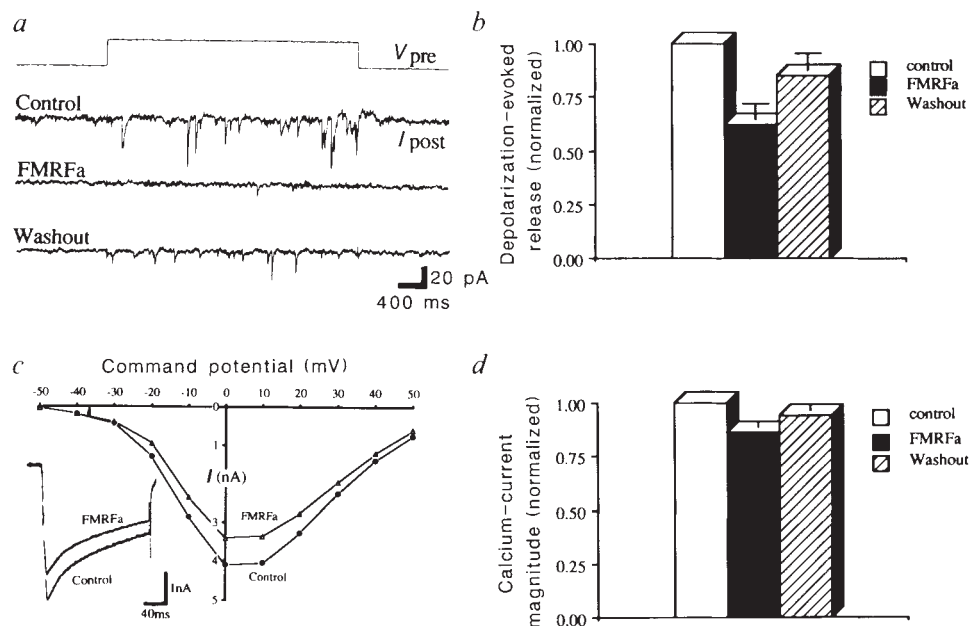
Buccal ganglion neuron B5 forms cholinergic chemical connections with buccal neuron B19 in cell culture. Under culture conditions that prevent neurite extension, somata remain spherical, gain the ability to release ACh from the entire soma surface and form chemical synapses with contacting somata^{1,2}. We monitored postsynaptic current (p.s.c.) in cholinceptive neuron B19 using patch pipettes in the whole-cell mode as described previously³. To examine the presynaptic actions of FMRFa on ACh release, the presynaptic neuron B5 was depolarized for 4 s from a holding potential of -60 mV to evoke a desynchronized release of neurotransmitter (Fig. 1a). This release of neurotransmitter is both calcium-dependent and reversibly reduced by the cholinergic antagonist, tubocurarine¹.

The number of p.s.c.s evoked during 4-s depolarizing steps was counted in control conditions, in the presence of 1 μ M FMRFa and after washout. FMRFa reliably reduced the number of depolarization-evoked p.s.c.s ($n=9$; Fig. 1a), but had little effect on the postsynaptic sensitivity to ACh (data not shown). The number of p.s.c.s were normalized to a value of 1.0, which corresponded to the number of p.s.c.s in the control period. FMRFa reduced the depolarization-evoked ACh release to 0.62 ± 0.07 of that of the control (mean $\pm$ s.e.m.; Fig. 1b). This reduction of ACh release was due to an action of FMRFa and not to synaptic fatigue, because the depolarization-evoked release of ACh was increased, after washout, to 0.85 ± 0.08 of that of the control (Fig. 1b; $P < 0.025$, two-tailed Student's t -test,

FIG. 1 FMRFa causes a presynaptic inhibition of ACh release and a reduction in the macroscopic calcium current.

a, Spherical secretory neuronal somata were voltage clamped in the whole-cell configuration. The presynaptic secretory soma was depolarized (V_{pre}) from -60 mV to -15 mV to cause a desynchronized release of ACh, which is seen as a train of inward (downward) p.s.c.s in the current recordings from the postsynaptic cell (I_{post}). Application of FMRFa (1 μ M) reversibly reduced the number of desynchronized p.s.c.s (synapse R03). **b**, Histograms demonstrating the reversible reduction in ACh release evoked by FMRFa ($n=9$). The presynaptic soma was repetitively depolarized for 4 s at 30 s intervals. The average number of p.s.c.s evoked during three trials is represented in each experimental category (mean $\pm$ s.e.m.). Values are normalized (see text). **c**, Current-voltage relationship of the HVA calcium current. FMRFa did not change the extrapolated positive reversal potential of the calcium current (neuron C21). The macroscopic calcium current of a secretory soma (inset) is shown in control conditions and after application of 1 μ M FMRFa (neuron C36; holding potential -60 mV; command potential 10 mV). **d**, Histograms demonstrating the reversible inhibitory action of FMRFa on the HVA calcium current of secretory somata ($n=18$). The presynaptic soma was depolarized repetitively for 200 ms at intervals of 30 s. The average peak current during three trials is represented in each experimental category (mean $\pm$ s.e.m.). Values are normalized.

METHODS. Neurons B5 and B19 were isolated from buccal ganglia of adult specimens of *Helisoma* and were plated together in freshly-collected snail haemolymph (10%) in 50% salt-free Leibowitz culture medium (GIBCO) plus 40 mM NaCl, 1.7 mM KCl, 4.1 mM CaCl₂, 1.5 mM MgCl₂, 10 mM K⁺-HEPES (Sigma), pH 7.3, and 50 μ g ml⁻¹ gentamicin sulphate (Sigma)^{1,2}. Snail haemolymph plasma prevented cell adhesion to the culture dish and dis-



couraged neurite extension. Culture medium was changed daily. After 2 days, synaptic partners were transferred to poly-L-lysine-coated Petri dishes for immobilization and electrophysiological examination. Neurons were voltage-clamped using Dagan Corporation 8900 patch amplifiers. Postsynaptic pipettes contained 50 mM KCl, 5 mM MgCl₂, 5 mM EGTA and 5 mM K⁺-HEPES, pH 7.3. Presynaptic neuron B5 was dialysed with a similar internal solution, which contained 35 mM CsCl instead of KCl plus 5 mM ATP and 1 mM GTP. When calcium currents were examined, neurons were bathed in 0 mM NaCl, 4.1 mM CaCl₂, 1.5 mM MgCl₂, 1.7 mM KCl, 10 mM 4-aminopyridine, 30 mM tetraethylammonium bromide, 30 mM sucrose, 10 mM K⁺-HEPES, pH 7.3. Linear leakage and capacity currents were digitally subtracted from depolarizing current traces using pClamp software (Axon Instruments, California).

$t = 2.41$, coefficient of variation (d.f. = 16, $n = 9$). Because FMRFa reduced the number of depolarization-evoked p.s.c.s, this agonist must have been causing a presynaptic inhibition of ACh release from neuron B5.

Neuromodulators can bring about their effects on evoked transmitter release by regulating the activity of voltage-dependent ion channels⁹⁻¹⁸. We voltage-clamped secretory somata of neuron B5 and studied the high-voltage activated (HVA) calcium current, which raises cytoplasmic calcium, which in turn triggers ACh release. In ionic and pharmacological conditions that permitted the selective detection of calcium currents², neuron B5 was depolarized from a holding potential of -60 mV to command potentials ranging from -50 to $+50$ mV. FMRFa ($1 \mu\text{M}$) reliably reduced the macroscopic current (Fig. 1c; $n = 22$). This effect was judged to be a reduction in calcium current, because FMRFa did not affect the extrapolated positive reversal potential of the calcium current (Fig. 1c; $n = 4$), and addition of the calcium-channel antagonist Cd^{2+} ($5 \mu\text{M}$; $n = 12$), which reduced the calcium-current magnitude, reduced the extent of the action of FMRFa.

We determined the magnitude of the HVA calcium current in three control depolarizing steps to constant command potentials spaced at intervals of 30 s. Cells were discarded from further analysis if the current decreased by $\geq 5\%$ during this period. FMRFa ($1 \mu\text{M}$) reduced HVA calcium current to 0.86 ± 0.03 (Fig. 1d; $n = 18$) of the control calcium current. After washout the current significantly increased to 0.94 ± 0.03 of the control current ($P < 0.025$, $t = 2.08$, d.f. = 34, $n = 18$ cells).

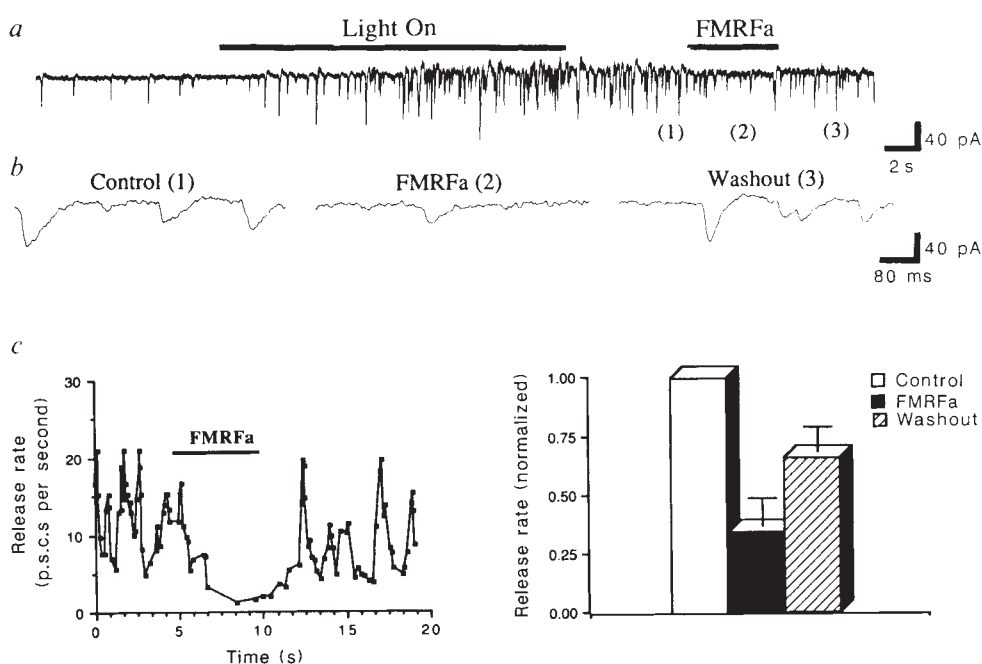
In non-neuronal cells, the sensitivity of the secretory apparatus to internal calcium is regulated by multiple intracellular signals¹⁹⁻²². Thus, we determined whether FMRFa additionally regulates the Ca^{2+} -dependent secretory apparatus. Because FMRFa affects the calcium current and thus calcium influx, it was necessary to control the internal free-calcium so that it was independent of transmembrane ion fluxes. We used the photolabile calcium cage, nitr-5 (Calbiochem)³⁻⁸, to elevate the internal level of free calcium and evoke transmitter release,

thereby dissociating voltage-dependent calcium current from the calcium-dependent secretory event. Nitr-5 (20 mM) loaded to 90% of its full capacity with $18 \text{ mM } \text{Ca}^{2+}$, was included in the internal patch-pipette solution in place of EGTA. Characteristically, after 1–5 min of presynaptic recording, the background rate of p.s.c.s increased, indicating successful dialysis (estimated pre-photolysis calcium level $\sim 400 \text{ nM}$). Exposure to ultraviolet light (340 nm) photolytically released calcium from the cage and caused a sustained increase in the rate of p.s.c.s (Fig. 2a; $n = 11$). These p.s.c.s were judged to be due to ACh because $10 \mu\text{M}$ tubocurarine reversibly reduced their magnitude. Ultraviolet light did not increase the background rate of p.s.c.s when cells were dialysed with nitr-5 that had not been loaded with calcium ($0 \text{ mM } \text{Ca}^{2+}$, 20 mM nitr-5; $n = 4$).

We tested the effect of FMRFa on the secretory apparatus. Neurons were voltage clamped at -80 mV and the rate of calcium-dependent ACh release was increased by the photolytic release of calcium from nitr-5 (Fig. 2a). A puff of FMRFa (5–10 s) was applied to cell pairs. In all of nine applications (eight synapses), FMRFa (1 – $10 \mu\text{M}$) reversibly reduced the rate of p.s.c.s (Fig. 2). In an additional two preparations bathed in $0 \text{ mM } \text{Ca}^{2+}/10 \text{ mM } \text{Mg}^{2+}$ saline, FMRFa had the same effect. The rate of transmitter release was quantified by calculating the reciprocal of the interval between successive p.s.c.s. Because transmitter release has a statistical nature, the measured rate of transmitter release was smoothed using the moving bin method^{3,23} over three events. Figure 2c shows that FMRFa reversibly reduced the rate of transmitter release. In Fig. 2d, normalized data from all cell pairs examined are shown. FMRFa reduced the average rate of p.s.c.s to 0.35 ± 0.11 of the control rate, and after washout the rate significantly increased to 0.66 ± 0.1 of the control value ($P < 0.025$, $t = 2.15$, d.f. = 16, $n = 9$).

Because nitr-5 is probably the dominant calcium buffer in the dialysed cell we expected free calcium to be unaffected by FMRFa. In four control experiments this was confirmed using fura-2 (refs 7, 24). Calcium was photolytically released from nitr-5 to yield an estimated free-calcium level of $\sim 1 \mu\text{M}$. Appli-

FIG. 2 FMRFa reduces the rate of p.s.c.s under conditions of constant internal calcium. Whole-cell recordings were made from presynaptic neuron B5 using patch pipettes that contained 20 mM nitr-5, $18 \text{ mM } \text{CaCl}_2$, $35 \text{ mM } \text{KCl}$, $5 \text{ mM } \text{MgCl}_2$, $5 \text{ mM } \text{ATP}$, $1 \text{ mM } \text{GTP}$, $400 \mu\text{M}$ fura-2, $5 \text{ mM } \text{K}^+$ -HEPES, pH 7.3. The release of neurotransmitter was detected as inward current transients (p.s.c.s). a, Epi-illumination with ultraviolet light, supplied by a xenon-arc lamp through the $\times 40$ oil-immersion objective of a Nikon Diaphot inverted microscope, photolytically released calcium and triggered an increase in p.s.c. frequency. After ceasing illumination, ACh release was sustained. Subsequent application of $1 \mu\text{M}$ FMRFa in 0.1% fast green in culture medium from a puffer pipette decreased the rate of p.s.c.s. b, Expanded records of the p.s.c.s in control conditions (1), during the 5-s FMRFa puff (2) and after washout (3). P.s.c.s were readily detected above the noise levels of the recordings (synapse N13 in a and b). c, Rate of p.s.c.s (see text for method of calculation) in response to elevated internal calcium. Puffer application of FMRFa ($10 \mu\text{M}$) rapidly reduced the rate of ACh release. At the end of FMRFa application, an initial return towards the control rates of p.s.c.s was detected within seconds of washout (synapse N17). d, Collective data showing the normalized rate of p.s.c.s. The average rate of release was calculated for each synapse over 3-s periods immediately before FMRFa



cation (control), during the last 3 s of FMRFa application and during 2–5 s of washout. FMRFa reliably reduced the rate of ACh release. Partial recovery during the washout period may be due to incomplete washout of the agonist. The larger inhibitory effect of FMRFa on transmitter release evoked by caged calcium as compared with transmitter release evoked by depolarization (Fig. 1b) was probably due to different doses of FMRFa.

cation of FMRFa (10 μ M) did not change this level of free calcium in nitr-5-loaded presynaptic cells ($n = 4$). Thus, FMRFa reversibly decreased the rate of calcium-dependent ACh release under conditions of constant and elevated calcium.

There are numerous potential mechanisms that could bring about FMRFa's action on the secretory apparatus. In squid, Ca^{2+} /calmodulin-dependent protein kinase II, which phosphorylates the vesicle-associated protein synapsin I (ref. 25), increases the release of transmitter without affecting the initial macroscopic calcium-current²⁶. It is postulated that phosphorylation increases the number of vesicles available for transmitter release thereby causing synaptic facilitation. In *Helisoma*, FMRFa might have an opposing action, promoting a dephosphorylation of vesicle proteins to reduce the number of vesicles available for transmitter release, thereby contributing to presynaptic inhibition.

The neuropeptide FMRFa, therefore, acts through multiple routes to bring about a presynaptic inhibition of transmitter release. First, FMRFa reduces the HVA calcium current of the secretory membrane. Second, FMRFa decreases the rate of ACh release in response to a constant level of presynaptic internal free-calcium. This is a unique observation in neuronal tissue. Because intracellular signals commonly regulate the sensitivity of the secretory apparatus to free calcium in non-nervous secretory tissue¹⁹⁻²², it will be intriguing to determine whether this is a widespread regulatory mechanism used throughout synaptic terminals. $\square$

Received 24 April; accepted 11 August 1989.

- Haydon, P. G. *J. Neurosci.* **8**, 1032-1038 (1988).
- Haydon, P. G. & Man-Son-Hing, H. *J. Neurosci.* **1**, 919-927 (1988).
- Zucker, R. S. & Haydon, P. G. *Nature* **335**, 360-362 (1988).
- Tsien, R. Y. & Zucker, R. S. *Biophys. J.* **50**, 843-853 (1986).
- Gurney, A. M., Tsien, R. Y. & Lester, H. A. *Proc. natn. Acad. Sci. U.S.A.* **84**, 3496-3500 (1987).
- Adams, S. R., Kao, J. P. Y., Grynkiewicz, G., Minta, A. & Tsien, R. Y. *Am. chem. Soc.* **110**, 3212-3220 (1988).
- Tsien, R. Y. *Trends Neurosci.* **11**, 419-424 (1988).
- Kaplan, J. H. & Somolyo, A. P. *Trends Neurosci.* **12**, 54-59 (1989).
- Kandel, E. R. & Schwartz, J. H. *Science* **218**, 433-443 (1982).
- Klein, M., Camardo, J. S. & Kandel, E. R. *Proc. natn. Acad. Sci. U.S.A.* **79**, 5713-5717 (1982).
- Siegelbaum, S. A., Camardo, J. S. & Kandel, E. R. *Nature* **299**, 413-417 (1982).
- Tsien, R. W., Lipscombe, D., Madison, D. V., Bley, K. R. & Fox, A. P. *Trends Neurosci.* **11**, 431-438 (1988).
- Ewald, D. A., Pang, I.-H., Sternweis, P. C. & Miller, R. J. *Neuron* **2**, 1185-1193 (1989).
- Harris-Warrick, R. M. et al. *Neuron* **1**, 27-32 (1988).
- Holz, G. G., Rane, S. G. & Dunlap, K. *Nature* **319**, 670-672 (1986).
- Colombaloni, L., Paupardin-Trisch, D., Vidal, P. P. & Gerschenfeld, H. M. *J. Neurosci.* **5**, 2533-2538 (1985).
- Brezina, V., Eckert, R. & Erxleben, C. *J. Physiol., Lond.* **388**, 565-595 (1987).
- Kramer, R. H., Levitan, E. S., Carrow, G. M. & Levitan, I. B. *J. Neurophysiol.* **60**, 1728-1737 (1988).
- Baker, P. *Prog. Zool.* **33**, 265-274 (1986).
- Knight, D. E. & Scrutton, M. C. *Nature* **309**, 66-68 (1984).
- Lohse, M. J., Klotz, K.-N., Salzer, M. J. & Schwabe, U. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8875-8879 (1988).
- Knight, D. E. & Baker, P. F. *Membr. Biol.* **68**, 107-140 (1982).
- Rahamimoff, R. & Yaari, Y. *J. Physiol., Lond.* **228**, 241-257 (1973).
- Grynkiewicz, G., Pionie, M. & Tsien, R. Y. *J. Biol. Chem.* **260**, 3440-3450 (1985).
- Nestler, E. J. & Greengard, P. *Protein Phosphorylation in the Nervous System* (Wiley, New York, 1984).
- Llinas, R. et al. *Proc. natn. Acad. Sci. U.S.A.* **82**, 3035-3039 (1985).

ACKNOWLEDGEMENTS. We thank Drs C. Drewes, J. Johansen, B. MacVicar and R. S. Zucker for their comments on the manuscript and to R. Doyle for his technical assistance. This work was supported by the NIH and the Iowa State University Biotechnology Council (P.G.H.) and the MRC of Canada and a NATO International Collaboration Fellowship (K.L.). P.G.H. is an Alfred P. Sloan Fellow.

Caenorhabditis elegans has scores of homoeobox-containing genes

Thomas R. Bürglin, Michael Finney, Alan Coulson* & Gary Ruvkun

Department of Molecular Biology, Wellman 8, Massachusetts General Hospital and Department of Genetics, Harvard Medical School, Boston, Massachusetts 02114, USA

*MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

HOMEOBOX-containing genes control cell identities in particular spatial domains, cell lineages, or cell types during the development of *Drosophila*^{1,2} and *Caenorhabditis elegans*³⁻⁵, and they probably control similar processes in vertebrates⁶⁻⁹. More than 80 genes with homoeoboxes that have sequence similarities ranging from 25 to 100% have been isolated by genetic means or by DNA hybridization to previously isolated genes¹⁰. We synthesized 500-2,000-fold degenerate oligonucleotides corresponding to a set of well-conserved eight amino acid sequences from the helix-3 region of the homoeodomain¹¹. We screened *C. elegans* genomic libraries with these probes and identified 49 putative homoeobox-containing loci. DNA sequencing confirmed that eight out of ten selected loci had sequences corresponding to the conserved helix-3 region¹¹ plus additional flanking sequence similarity. One of these genes contained a sequence corresponding to a complete *pou*-domain¹² and another was closely related to the homoeobox-containing genes *caudal/cdx-1*^{13,14}. The putative homoeobox loci were mapped to the physical contig map of *C. elegans*^{15,16}, allowing the identification of potentially corresponding genes from the correlated genetic map. We estimate that the number of homoeobox-containing genes in *C. elegans* is at least 60, constituting ~1% of the estimated total number of genes¹⁷.

An eight amino-acid degenerate motif from the helix-3 region of the homoeodomain is only found in homoeodomain proteins when the EMBL database is searched¹¹. To screen the *C. elegans*

genome for sequences corresponding to this conserved set of amino-acid sequences, we designed five 512-fold to 2,048-fold degenerate oligonucleotide probes (23 bases) corresponding to these amino-acid sequences (Fig. 1a). The HB-1 probe was designed to detect a large set of homoeobox-containing gene classes (for example, *Antp*, *Abd B*, *caudal*, *Dfd*, *lab*, *mab-5*, *ro*, *Ubx*, *zen-1*; see ref. 10) with no mismatches, and the *eve*, *en* and *bcd* classes¹⁰ with one mismatch. The PRD-1/PRD-2 set of oligonucleotides detects homoeoboxes of the *paired* class¹⁰ with no mismatches, whereas the POU-1/POU-2 set was designed to detect members of the *pou*-domain class with one or no mismatch¹². Statistical analysis indicates that with two or fewer mismatches, few spurious random hybridizations to these probes would be expected in the small *C. elegans* genome (100 million base pairs; J. Sulston and A.C., unpublished observation).

To establish the specificity of the degenerate oligonucleotides and the hybridization conditions we probed a panel of sequenced homoeobox-containing genes that had zero to seven mismatches with HB-1, PRD-1, and PRD-2 probes (Fig. 1b). We determined conditions that yielded strong hybridization to fragments with two or fewer mismatches. Using these conditions and probes, ~35 bands of varying intensities could be distinguished on a genomic Southern blot of *C. elegans* DNA (Fig. 1c).

We screened genomic cosmid libraries¹⁵ containing two to four genome equivalents with these probes and selected 136 cosmids. These cosmid DNAs were digested with *EcoRI*, transferred to Southern blots and hybridized separately with HB-1, PRD-1/PRD-2, and POU-1/POU-2. The cosmids were also fingerprinted and their positions in the 90-95%-complete physical map of the *C. elegans* genome^{15,16} were determined. Many of the isolated cosmids overlapped, so that finally 50 independent hybridizing loci were isolated (Table 1) and assigned to contigs as large as 8 megabases.

The contig map indicated that four loci that we had isolated mapped to the same cosmids as known homoeobox-containing genes. One locus (no. 47) was the known homoeobox-containing gene *unc-86* (ref. 5). Three loci (nos. 7, 20 and 31) coincided with homoeobox-containing genes isolated and sequenced by other groups: *ceh-1* and *ceh-11* (N. Hawkins and J. McGhee, personal communication) and *ceh-11* and *ceh-12* (D. Schaller,

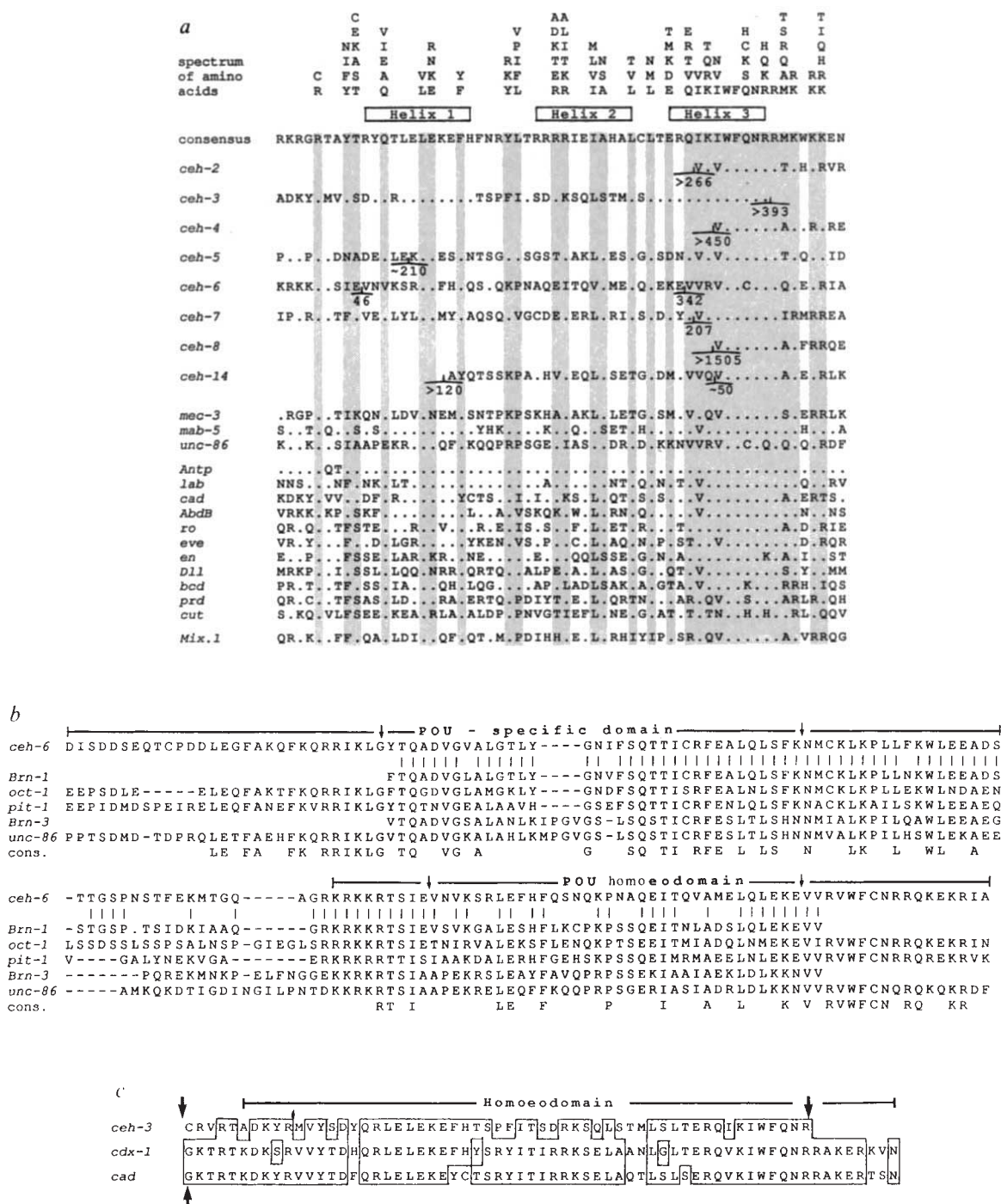


FIG. 2 *a*, Comparison of the deduced sequences of the homoeodomains of the *ceh-2* to *ceh-8* and *ceh-14* gene products and selected *C. elegans*, *Drosophila* and frog homoeodomains^{3,4,5,10,27,28}. Single-letter code designates amino-acid residues. Dots indicate identity to an arbitrary consensus¹¹. Amino acids listed above the consensus are the spectrum of residues observed at the least variable 30 positions of the homoeodomain¹⁰ (yeast homoeodomains not included). Note that for the *ceh* genes there are matches to these positions outside the eight matches corresponding to the oligonucleotide probes. Only one residue of the *ceh-3* sequence and one in *ceh-14* violate this rule. Helices are denoted according to Otting *et al.*²⁹. The *ceh* genes detected here hybridized with 0 to 3 mismatches to HB-1 or PRD-1/PRD-2. The spurious hybridizing clones nos. 7, 27 hybridized with an additional loopout. The genes *ceh-2* to *ceh-8* and *ceh-14* all have introns (positions indicated by arrows) in the homoeobox (sizes indicated underneath each line) based on genomic DNA sequences. The donor and acceptor sequences of these introns conform to the well-conserved *C. elegans* splice sites¹⁷. The position of the intron in *ceh-2* and *ceh-7* is the same as the position of the intron in the *Drosophila* genes *labial*¹⁰ and *Distal-less*²⁷.

whereas that in *ceh-4*, *ceh-8* and *ceh-14* is the same as in the frog gene *Xhox3* (ref. 30). *b*, Alignment of the deduced amino acid sequences of the *ceh-6*, *Brn-1*, *oct-1*, *pit-1*, *Brn-3* and *unc-86* gene products^{12,20}. That of *ceh-6* is 92% identical to that of *Brn-1* in the POU-specific domain, and 69% in the POU homoeodomain (vertical-dashes indicate identities). Arrows indicate introns in *ceh-6*. *c*, Alignment of the deduced homoeodomain sequences of the *ceh-3*, *caudal*¹² (*cad*) and *cdx-1* gene products¹³. The *ceh-3* partial homoeodomain is 67–69% identical to that of *cdx-1* and *cad*. Note the conservation of amino acids just upstream of the homoeodomain, and the similar location of the intron for *caudal* and *ceh-3* (indicated by arrows).

METHODS. Homoeobox-hybridizing *EcoRI* fragments were subcloned into Bluescribe⁻ (Stratagene) or M13mp18 using standard techniques²⁵ and sequencing was carried out using Sequenase (USB) according to manufacturer's instructions. In most cases some initial sequence was obtained using the degenerate oligonucleotides as primers. Sequences were extended by synthesizing specific primers.

Loci no.	Cosmid name	No. of ind. clones	Probe	Chromosome	Gene	Loci no.	Cosmid name	No. of ind. clones	Probe	Chromosome	Gene
1	AC5	2	HB-1	I	<i>ceh-5</i>	28	QB9	1	Weak HB-1	V	
2	DH6	5	PRD-1/-2	I	<i>ceh-6</i>	29	WWF9	4	HB-1	V	
3	XXD4	2	HB-1	I	<i>ceh-8</i>	30	XXF5	1	HB-1	X	<i>ceh-14</i>
			Weak HB-1			31	QQB7	2	Very weak HB-1	X	<i>ceh-1</i>
4	AB3	7	HB-1	I	<i>lin-11?</i>	32	JA2	1	HB-1	X	
5	CCCC5	1	HB-1	I		33	CB11	3	PRD-1/-2	X	
6	NE4	1	HB-1	I	<i>ceh-2</i>	34	QQQA5	1	HB-1	X	
7	DDDG7	3	HB-1	I	<i>ceh-12</i>	35	WH2	3	HB-1	X	
8	CF10	5	HB-1	II	<i>ceh-7</i>	36	C17D2	1	HB-1	X	
9	HH5	2	HB-1	II		37	BBBF1	1	HB-1	?	Unmapped contig
			Weak HB-1			38	EEF1	5	HB-1	?	Unmapped contig
10	IIIE1	1	HB-1	II		39	LLLC5	1	Very weak HB-1	?	Unmapped contig
11	RB9	1	Weak PRD-1/-2	II		40	NB4	2	PRD-1/-2	?	Unmapped contig
12	SC10	1	Weak PRD-1/-2	II		41	XC10	1	HB-1	?	Unmapped contig
13	VA4	1	Very weak HB-1	II					Weak HB-1		
14	SD6	3	HB-1	II	<i>ceh-4, unc-4?</i>	42	IID7	1	HB-1	?	No match
15	XD3	1	HB-1	II		43	KB3	1	HB-1	?	No match
16	AC10	2	Weak HB-1	III	<i>ceh-3</i>						
17	AAAC1	2	HB-1	III	No homoeobox						
18	CH5	4	HB-1	III		44	C03D6	1	POU-1/-2	I	
			Weak HB-1			45	C30G1	1	Weak POU-1/-2	I	
19	JG10	6	HB-1	III		46	C37A2	1	POU-1/-2	I	
20	C27D11	1	HB-1	III	<i>ceh-11</i>	2	C47D1	2	POU-1/-2 & POU5P-A	I	<i>ceh-6</i>
21	KB2	2	HB-1	IV							
22	NA6	1	Weak HB-1	IV		47	C454G4	2	POU-1/-2 & POU5P-A	III	<i>unc-86</i>
23	VA2	1	Very weak HB-1	IV							
24	AH1	3	HB-1	V		48	C56D1	1	POU-1/-2	V	
25	ID10	2	HB-1	V		49	C52F10	2	POU-1/-2	X	
26	NA2	2	HB-1	V					POU-1/-2		
27	PPA6	1	HB-1	V	No homoeobox				POU-1/-2		
						50	C46E7	1	POU-1/-2	?	Unmapped contig

Figure 1: Genetic map of the *unc-104* locus on Chromosome II. The map shows the arrangement of genes and markers on Chromosomes I, II, III, IV, V, and X. Chromosome I contains *unc-38*, *dpy-5*, *hP5*, *SP1*, *unc-15*, *unc-29*, *lin-10*, *hP6*, *lin-11*, and *ceh-5*. Chromosome II contains *dpy-10*, *unc-104*, *TCM15A*, *TCMUT5B*, *rol-6*, *unc-4*, *eP44*, *TCM129*, and *sqt-1*. Chromosome III contains *unc-93*, *ced-4*, *ceh-3*, *mab-5*, and *unc-86*. Chromosome IV contains *col-4*, *fem-3*, *stP1*, *mec-3*, and *unc-22*. Chromosome V contains *her-1*, *act-1*, *col-12*, *rsn-4*, and *unc-28*. Chromosome X contains *act-4*, *vit-3*, *vit-2*, *unc-6*, *unc-18*, *xol-1*, *abi-1*, *lin-14*, *np8*, and *ceh-14*. A 2Mb scale bar is shown at the bottom right.

NATURE · VOL 341 · 21 SEPTEMBER 1989

pou-IV subclasses¹⁸ (*ceh-6*, *unc-86*), suggests that these, and probably many of the other classes, were already present in the common ancestor of *C. elegans*, *Drosophila* and vertebrates. The new classes of homoeobox-containing genes so far found only in *C. elegans* (*mec-3* and *ceh-14*, *ceh-5*, *ceh-7*) may also be present but as yet undetected in vertebrates and other invertebrates.

Many of the homoeobox loci were mapped with a resolution of 30–50 kilobases to large contigs allowing their location relative to each other to be determined (Fig. 3). These genes seemed to be randomly distributed along the contigs; no clusters of homoeobox-containing genes like those in *Drosophila* and vertebrates^{2,20,21} were detected. Some cosmids (nos. 3, 9, 18, 41 and 49; Table 1) contained several hybridizing bands and thus possibly more than one homoeobox gene.

About 125 genetic markers have similarly been mapped to the *C. elegans* contig map, thus aligning large stretches of the genetic and physical maps. The mapping of homoeobox-containing genes within these contigs immediately suggested potential genes to which they may correspond (Fig. 3). One of the loci mapped to a cosmid that contained the *lin-11* gene, which controls cell identities in some vulval precursor cells²² (G. Freyd, S. Kim and H. R. Horvitz, personal communication). Likewise, *ceh-4* mapped to the same cosmid that contained allele-specific polymorphisms in the neural identity gene *unc-4* (D. Miller, personal communication). Many of the other loci identified here may not have been identified by mutation. Their location on the genetic map can be used to obtain mutations in these genes in a directed fashion.

Because eight of the ten putative homoeobox loci that we sequenced, including a weakly hybridizing locus, actually contained a homoeobox, and because four of the loci we detected were confirmed by others, we expect that most (~85%) of the 36 other loci isolated are homoeobox-containing genes. Poisson analysis of the number of times each locus was isolated predicts that for statistical reasons we should have missed ~20 loci that could be represented in cosmid libraries. Indeed, we failed to detect cosmids containing *mab-5*⁴ and *mec-3*³, both of which should have been detected by our probes. A conservative estimate for the number of homoeobox-containing genes in *C. elegans* that our probes would detect (see Table 1) is thus 60, or ~1% of the estimated total number of genes¹⁷.

Clone arrays similar to that for *C. elegans* are being constructed for many organisms as chromosomal walks towards genetic loci or as a prelude to genome sequencing. Whereas the complete DNA sequence of such arrays may be years away, the identification and mapping of particular sequence motifs (such as zinc-fingers, kinases), as we have shown here with the homoeodomain, can highlight specific genetic regions for further study. □

Received 21 June; accepted 8 August 1989.

1. Akam, M. *Development* **101**, 1–22 (1987).
2. Gehring, W. J. *Science* **236**, 1245–1252 (1987).
3. Way, J. C. & Chalfie, M. *Cell* **54**, 5–16 (1988).
4. Costa, M., Weir, M., Coulson, A., Sulston, J. & Kenyon, C. *Cell* **55**, 747–756 (1988).
5. Finney, M., Ruvkun, G. & Horvitz, H. R. *Cell* **55**, 757–769 (1988).
6. Cho, K. W. Y. *et al.* *EMBO J.* **7**, 2139–2149 (1988).
7. Harvey, R. P. & Melton, D. A. *Cell* **53**, 687–697 (1988).
8. Ruiz i Altaba, A. & Melton, D. A. *Cell* **57**, 317–326 (1989).
9. Wright, C. V. E., Cho, K. W. Y., Hardwicke, J., Collins, R. H. & De Robertis, E. M. *Cell* (in the press).
10. Scott, M. P., Tamkun, J. W. & Hartzell III, G. W. *BBA reviews on cancer* Vol. 9, 25–48 (1989).
11. Bürglin, T. R. *Cell* **53**, 339–340 (1988).
12. Herr, W. *et al.* *Genes Dev.* **2**, 1513–1516 (1988).
13. Mlodzik, M. & Gehring, W. J. *Cell* **48**, 465–478 (1987).
14. Duprey, P. *et al.* *Genes Dev.* **2**, 1647–1654 (1988).
15. Coulson, A., Sulston, J., Brenner, S. & Karn, J. *Proc. Natn. Acad. Sci. U.S.A.* **83**, 7821–7825 (1986).
16. Coulson, A., Waterston, R., Kiff, J., Sulston, J. & Kohara, Y. *Nature* **335**, 184–186 (1988).
17. Emmons, S. W. in *The Nematode Caenorhabditis elegans* (ed. Wood, W. B.) 47–79 (Cold Spring Harbor Laboratory, New York, 1988).
18. He, X. *et al.* *Nature* **340**, 35–42 (1989).
19. Kamb, A., Weir, M., Rudy, B., Varmus, H. & Kenyon, C. *Proc. Natn. Acad. Sci. U.S.A.* **86**, 4372–4376 (1989).
20. Graham, A., Papalopolu, N. & Krumlauf, R. *Cell* **57**, 367–378 (1989).
21. Duboule, D. & Dollé, P. *EMBO J.* **8**, 1497–1505 (1989).
22. Ferguson, E. L., Sternberg, P. W. & Horvitz, H. R. *Nature* **326**, 259–267 (1987).

23. Ausubel *et al.* *Current Protocols in Molecular Biology* (Green Publishing Associates and Wiley-Interscience, New York, 1987).
24. Rosenberg, U. B. *et al.* *Nature* **319**, 336–339 (1986).
25. Maniatis, T., Fritsch, E. F. & Sambrook, J. in *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).
26. Wood, W. I., Gitschier, J., Lasky, L. A. & Lawn, R. M. *Proc. Natn. Acad. Sci. U.S.A.* **82**, 1585–1588 (1985).
27. Cohen, S. M., Brönnner, G., Küttner, F., Jürgens, G. & Jäckle, H. *Nature* **338**, 432–434 (1989).
28. Rosa, F. M. *Cell* **57**, 965–974 (1989).
29. Otting, G. *et al.* *EMBO J.* **7**, 4305–4309 (1988).
30. Ruiz i Altaba, A. & Melton, D. A. *Development* **106**, 173–183 (1989).
31. Kramer, J. M., Johnson, J. J., Edgar, R. S., Basch, C. & Roberts, S. *Cell* **55**, 555–565 (1988).

ACKNOWLEDGEMENTS. We thank Joe Gatto for technical assistance, Mike Cherry for synthesizing oligonucleotides, and Rich Cate for discussions. We thank our colleagues for critical reading of the manuscript and G. Freyd, N. Hawkins, D. Miller and D. Schaller for exchange of data before publication. DNAs were gifts from Drs W. Gehring, M. Noll, C. Nüsslein-Volhard and G. Struhl. This work was supported by a Hoechst grant to the Massachusetts General Hospital, Department of Molecular Biology. T.B. is a recipient of an EMBO fellowship.

A Fos protein containing the Jun leucine zipper forms a homodimer which binds to the AP1 binding site

Manfred Neuberg, Jürgen Adamkiewicz,
John B. Hunter & Rolf Müller

Institut für Molekularbiologie und Tumorforschung (IMT), Philipps-Universität Marburg, Emil-Mannkopff-Strasse 2, D-3550 Marburg, FRG

THE TPA (12-*O*-tetradecanoyl-phorbol-13-acetate) responsive element (TRE) is recognized by the inducible transcription factor AP1, a heterodimeric complex of Fos- and Jun-protein subunits, which each contain a specific structure known as the leucine zipper through which they interact^{1–7}. Studies using site-directed mutagenesis have shown that a basic region adjacent to the leucine zipper in Fos is crucial for the interaction of the Fos–Jun complex with the TRE, and probably represents a site of interaction with DNA. The functionally crucial amino acids in this region are almost completely conserved between Fos and Jun (refs 6, 7 and 11; M.N. and R.M., unpublished results), indicating the formation of a nearly symmetrical DNA-binding site in the Fos–Jun complex. Whereas Jun can form a homodimeric protein complex which binds to the TRE, Fos is unable to do so. The Fos–Jun heterodimer, however, possesses at least a 30-fold-higher affinity for the TRE than does the Jun–Jun homodimer, indicating cooperative binding^{6–11}. Because Fos cannot form a homodimer it is not known whether Fos specifically recognizes part of the TRE or has a different role in the binding of the Fos–Jun complex to DNA. Here we report that exchanging the leucine zipper in Fos with that of Jun generates a protein (termed Ψ -Fos) that can form a complex with Fos. This Fos– Ψ -Fos complex, and to a lesser extent a homodimeric Ψ -Fos complex, exhibits specific binding to the TRE. This finding strongly supports the hypothesis that Fos and Jun form a nearly symmetrical DNA-binding site that interacts with the palindromic TRE.

To engineer a recombinant Fos protein that was able to form a homodimeric DNA-binding site we first introduced two new restriction sites into the *fos* gene by site-directed mutagenesis (without altering the coding capacity) at the 5'- and 3' ends of the leucine-zipper-encoding DNA sequences. The entire Fos zipper could then be exchanged with a double-stranded synthetic oligonucleotide representing the leucine repeat of Jun (see Fig. 1). The Ψ -Fos protein encoded by this gene differs from Fos only in the 24 amino acids that space the leucine residues. The Ψ -Fos protein was tested for its ability to form a pseudo-homodimer with Fos in an *in vitro* complex-reconstitution assay⁵. The formation of such a complex was analysed by using a Fos protein truncated at its C-terminal end, which enabled the two constituents to be distinguished by their differential

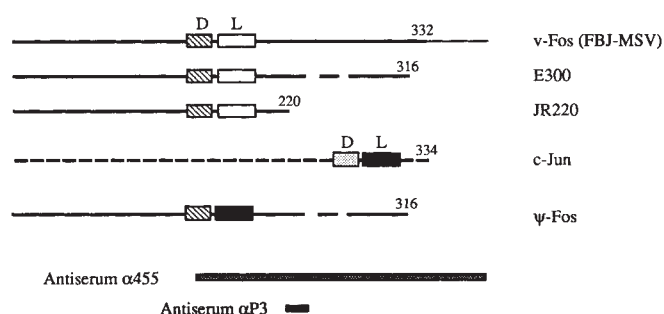


FIG. 1 Structure of Fos and Jun proteins encoded by the constructs used in this study. Numbers indicate amino acids; D, DNA-binding sites in Fos and Jun; L, leucine zippers in Fos and Jun; bold lines, Fos sequences; thin lines, v-Fos (FBJ-MSV)-specific sequences (that is, different from c-Fos sequences because of a frame-shift deletion¹⁶); dashed bold lines, Jun sequences. The stippled boxes at the bottom show those regions in Fos representing the antigens used to produce the antibodies $\alpha 455$ and $\alpha P3$. METHODS. Two restriction sites (*SacI* and *SphI*) were introduced by site-directed mutagenesis^{5,11} at nucleotide positions 2,418 and 2,457 (ref. 13) of E300. The leucine-zipper regions could thus be excised with *SacI* and *SphI* and be replaced with a synthetic double-stranded oligonucleotide encoding the Jun leucine zipper. Details of the other constructs have previously been published^{5,11,15}. Antiserum $\alpha P3$ was raised in a rabbit against a conjugate of thyroglobulin and a synthetic oligopeptide representing Fos amino acids 214 to 226 by using a water-soluble carbodiimide as the coupling agent¹⁷. Antiserum $\alpha 455$ has previously been described¹².

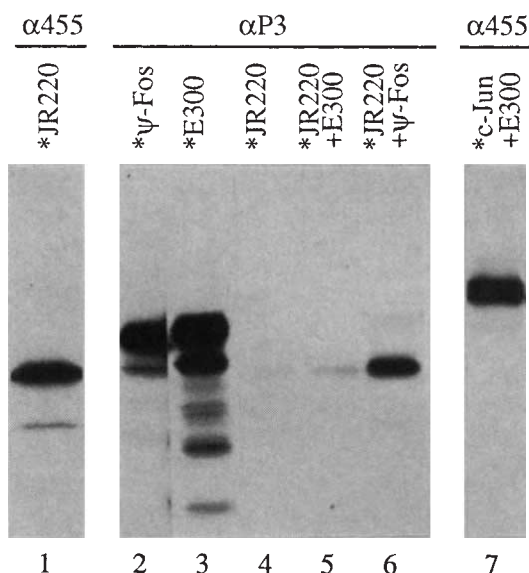


FIG. 2 *In vitro* protein-complex formation. The indicated *in vitro* translated proteins (asterisk denotes ³⁵S-methionine labelled proteins) were incubated either individually or together with another protein to allow complex formation as described⁵. The proteins were then immunoprecipitated by using the indicated antibodies and analysed by SDS-PAGE followed by fluorography. The weak bands in lanes 4 and 5 are due to a small degree of unspecific binding to the protein A used in the immunoprecipitation (data not shown).

interactions with antibody $\alpha P3$. This antibody was directed against amino acids 214–226 of Fos and so was able to recognize the E300 and Ψ -Fos proteins (amino acids 1–316; see Fig. 1) very strongly, but not JR220 (amino acids 1–220; see Fig. 1; Fig. 2, compare lanes 3 and 4). JR220 was precipitated, however, by $\alpha 455$ (lane 1), an antiserum directed against bacterially expressed Fos (see Fig. 1)¹². When labelled JR220 was incubated with unlabelled E300 and precipitated with $\alpha P3$ antibodies no complex formation could be detected (Fig. 2; similar back-

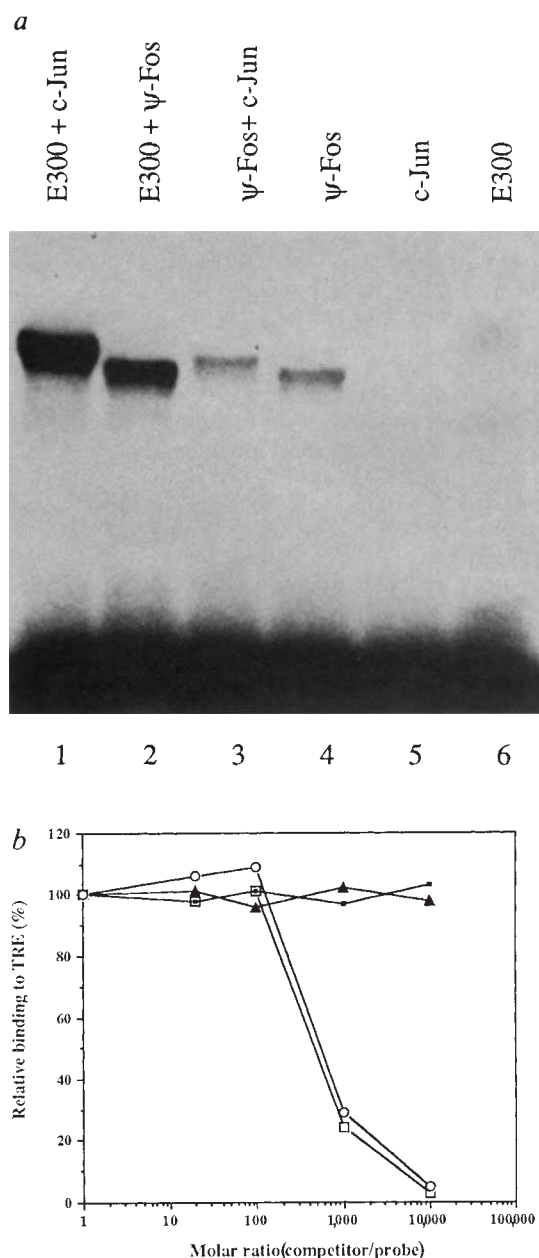


FIG. 3 *a*, TRE-binding properties of Fos, Ψ -Fos and Jun protein complexes. The indicated proteins were allowed to form complexes as in Fig. 2, incubated with a ³²P-labelled double-stranded oligonucleotide representing the collagenase TRE^{11,16} and analysed in a band shift assay^{18,19}. The free oligonucleotide is at the bottom of the gel. The weak bands in the Fos lane represent unspecific binding as shown by competition experiments (data not shown). *b*, Specificity of the Fos-Jun and Fos- Ψ -Fos complexes to the TRE. Binding of protein complexes to the TRE was determined after pre-incubation with different concentrations of either specific or non-specific oligonucleotides (for example, a TRE-containing oligonucleotide identical to the probe or an oligonucleotide of random sequence but the same size as the specific competitor). Complexes were analysed as in *a* and the relative percentage of binding was estimated by densitometric scanning of the autoradiogram (no competitor was taken as the 100% value). □, Fos-Jun + TRE; ○, Fos- Ψ -Fos + TRE; ■, Fos-Jun + random oligonucleotide; ▲, Fos- Ψ -Fos + random oligonucleotide.

ground bands in lanes 4 and 5). By contrast, complex formation was observed when labelled JR220 was incubated with Ψ -Fos (Fig. 2, lane 6). It seems that Fos (JR220) and Ψ -Fos associate less efficiently than Fos and Jun (Fig. 2; lane 7), although these results are not easily comparable because of the unavoidable use of different antibodies and *in vitro* translated proteins. These

observations demonstrate that it is the structure of the leucine zipper that normally prevents the formation of Fos homodimers. This may indicate that amino acids other than leucines have a role in the interaction.

The Ψ -Fos protein was then analysed for its ability to bind to the TRE as a homodimer or a pseudo-homodimer. The results of the band-shift assay are displayed in Fig. 3a: (1) under the conditions used no binding was observed with Fos (lane 6) or Jun (lane 5) alone, but the Fos-Jun complex (lane 1) bound cooperatively as previously reported (lanes 1-3). Under different experimental conditions, that is if the concentration of Jun protein or oligonucleotide probe is increased, it is possible to detect binding of Jun homodimers (ref. 9; also our unpublished observations); (2) the Fos- Ψ -Fos complex efficiently bound to the TRE oligonucleotide; and (3) both the Ψ -Fos homodimer and a Ψ -Fos-Jun heterodimer also bound to the TRE oligonucleotide, but with less affinity than the Fos- Ψ -Fos complex. (The formation of Ψ -Fos- Ψ -Fos and Ψ -Fos-Jun dimers has not been shown in a protein-complex reconstitution assay but is very likely because of the similar migration of the different protein-TRE complexes in the band-shift assay in Fig. 3a; that is, the migration in lane 3 is similar to that in lane 5, and that in lane 4 is similar to that in lane 6). The specificity of the binding of Fos- Ψ -Fos to the TRE was analysed in competition experiments using different concentrations of specific or non-specific oligonucleotides (Fig. 3b). Whereas no inhibition of binding was seen with the non-specific oligonucleotides, the specific TRE-containing oligonucleotide inhibited the binding of either the Fos-Jun complex or the Fos- Ψ -Fos complex to the TRE in a similar concentration-dependent manner. These results indicate that the Fos- Ψ -Fos complex can bind to the TRE with a relative affinity and specificity comparable to that of the binding of the Fos-Jun complex.

Our results demonstrate that the DNA-binding site of Fos can interact specifically with the TRE provided that a homodimeric complex is formed. It is probable that in the natural Fos-Jun complex the situation is similar. Our observations, therefore, strongly support the hypothesis that Fos and Jun form a nearly symmetrical DNA-binding site in which both proteins recognize similar nucleotides in the TRE. Each protein could thus interact with one half-site of the TRE. There could be at least two different reasons why the Fos- Ψ -Fos dimer binds with lower affinity to the TRE than does the Fos-Jun complex: (1) the association of Fos and Jun may be more stable than the Fos- Ψ -Fos complex as indicated by the results in Fig. 2; (2) the TRE is not perfectly symmetrical; only the seven core-bases form a perfect palindrome, but the sequence interacting with AP1 is longer, as indicated by mutagenesis of the TRE (ref. 20). Therefore, if one half-site of the TRE is preferred by Fos and the other by Jun, one would expect the Fos-Jun complex to show a higher affinity for the TRE than either the Fos- Ψ -Fos complex or the Jun-Jun complex.

The analysis of the interaction of AP1 with the TRE, and *trans*-regulation of AP1-dependent transcription is made complicated by the fact that AP1 is a heterodimer. In this respect the Ψ -Fos described here may prove very useful because it does not require Jun for specific DNA binding. □

14. Ryseck, R.-P., Hiaria, S. I., Yaniv, M. & Bravo, R. *Nature* **334**, 535-539 (1988).
15. Jenuwein, T. & Müller, R. *Cell* **48**, 647-657 (1987).
16. Van Beveren, C. *et al. Cell* **32**, 1241-1255 (1983).
17. Adamson, E. D., Meek, J. & Edwards, S. A. *EMBO J.* **4**, 941-947 (1985).
18. Angel, P. *et al. Cell* **49**, 729-739 (1987).
19. Barberis, A., Saperti-Furga, G. & Busslinger, M. *Cell* **50**, 347-359 (1987).
20. Risse, G., Joos, K., Neuberger, M., Brüller, H. J. & Müller, R. *EMBO J.* **8** (in the press).

ACKNOWLEDGEMENTS. We are grateful to Drs R.-P. Ryseck and R. Bravo for providing the *c-jun* cDNA clone, to Dr F. C. Lucibello for critically reading the manuscript, to Dr H. Brüller for synthesis of oligonucleotides and to U. Koch-Ei Abed for help in preparation of the manuscript.

Adaptive eradication of methionine and cysteine from cyanobacterial light-harvesting proteins

Didier Mazel & Philippe Marlière*

Unité de Physiologie Microbienne and *Unité de Biochimie Cellulaire, Département de Biochimie et Génétique Moléculaire, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France

SULPHUR is unique among the main elements of living cells in that it is covalently bound to biopolymers but does not occur in the biopolymer backbone. Indeed, most of the bacterial sulphur content resides in the methionine and cysteine side-chains of proteins¹. The growth yield of an organism under conditions of sulphur limitation could therefore be greatly enhanced by mutations that substitute Met and Cys in the organism's proteins for sulphur-free amino acids. Because the saving in sulphur would increase with such accumulating mutations, Met and Cys changes could be progressively selected. Abundant proteins should be the prime targets of such a selection. A few published observations give credence to this scenario. Sulphate permease, which is abundantly produced by sulphur-starved *Salmonella typhimurium*, lacks Met and Cys residues². Also, two species of marine purple bacteria synthesize more protein than can be expected from a limited sulphate supply³. We now report that the cyanobacterium *Calothrix* sp. PCC 7601 (referred to here as *Calothrix*) encodes sulphur-depleted versions of its most abundant proteins—phycocyanin and its auxiliary polypeptides—which it specifically expresses under conditions of sulphur limitation. Although these proteins do not take part in the fixation of sulphur, their elevated synthesis affects the sulphur budget of cyanobacterial cells. Direct evidence is thus provided that the structure of macromolecules can be subject to metabolic optimization.

Phycobilisomes, the light-harvesting complexes of cyanobacteria, account for up to 60% of the soluble protein content in these organisms⁴. The overall design of the phycobilisome consists of a hemidisk of six rods radiating fanwise from a central core^{5,6}. Phycobilisomes are made of three principal classes of chromophoric proteins (phycobiliproteins): allophycocyanin (AP) in the core, and phycocyanin (PC) and phycoerythrin (PE) in the rods, all composed of α - and β -subunits. All these chains ultimately derive from a unique common ancestor, structurally similar and evolutionarily related to the globin family⁷. Open tetrapyrrole chromophores (phycobilins) are post-translationally grafted to phylogenetically conserved Cys residues on both subunits⁸. In addition, phycobilisomes contain auxiliary proteins devoid of chromophore, called the linker polypeptides (LP). Three phycocyanin operons have been characterized in *Calothrix*⁸. The operon *cpc1*, which encodes the two PC1 subunits, is expressed regardless of the light spectrum, whereas transcription of the operon *cpc2*, which encodes the two PC2 subunits and their three associated linker polypeptides, is triggered by the red wavelength component⁸. In addition, a third operon, named *cpc3*, whose products had never been

Received 6 July; accepted 11 August 1989

1. Curran, T. & Franza, B. R. Jr *Cell* **55**, 395-397 (1988).
2. Landschulz, W. H., Johnson, P. F. & McKnight, S. L. *Science* **240**, 1759-1764 (1988).
3. Kouzarides, T. & Ziff, E. *Nature* **336**, 646-651 (1988).
4. Sassone-Corsi, P., Ransone, L. J., Lamph, W. W. & Verma, I. M. *Nature* **336**, 692-695 (1988).
5. Schuermann, M. *et al. Cell* **56**, 507-516 (1989).
6. Gentz, R., Rauscher, F. J. III, Abate, C. & Curran, T. *Science* **243**, 1695-1699 (1989).
7. Turner, R. & Tjian, R. *Science* **243**, 1689-1694 (1989).
8. Halazonetis, T. D., Georgopoulos, K., Greenberg, M. E. & Leder, P. *Cell* **55**, 917-924 (1988).
9. Rauscher, F. J. III, Voulalas, P. J., Franza, R. Jr, & Curran, T. *Genes Dev.* **2**, 1687-1699 (1988).
10. Nakabeppu, Y., Ryder, K. & Nathans, D. *Cell* **55**, 907-915 (1988).
11. Neuberger, M., Schuermann, M., Hunter, J. B. & Müller, R. *Nature* **338**, 589-590 (1989).
12. Verrier, B., Müller, D., Bravo, R. & Müller, R. *EMBO J.* **5**, 913-917 (1986).
13. Van Beveren, C., Enami, S., Curran, T. & Verma, I. M. *Virology* **135**, 229-234 (1984).

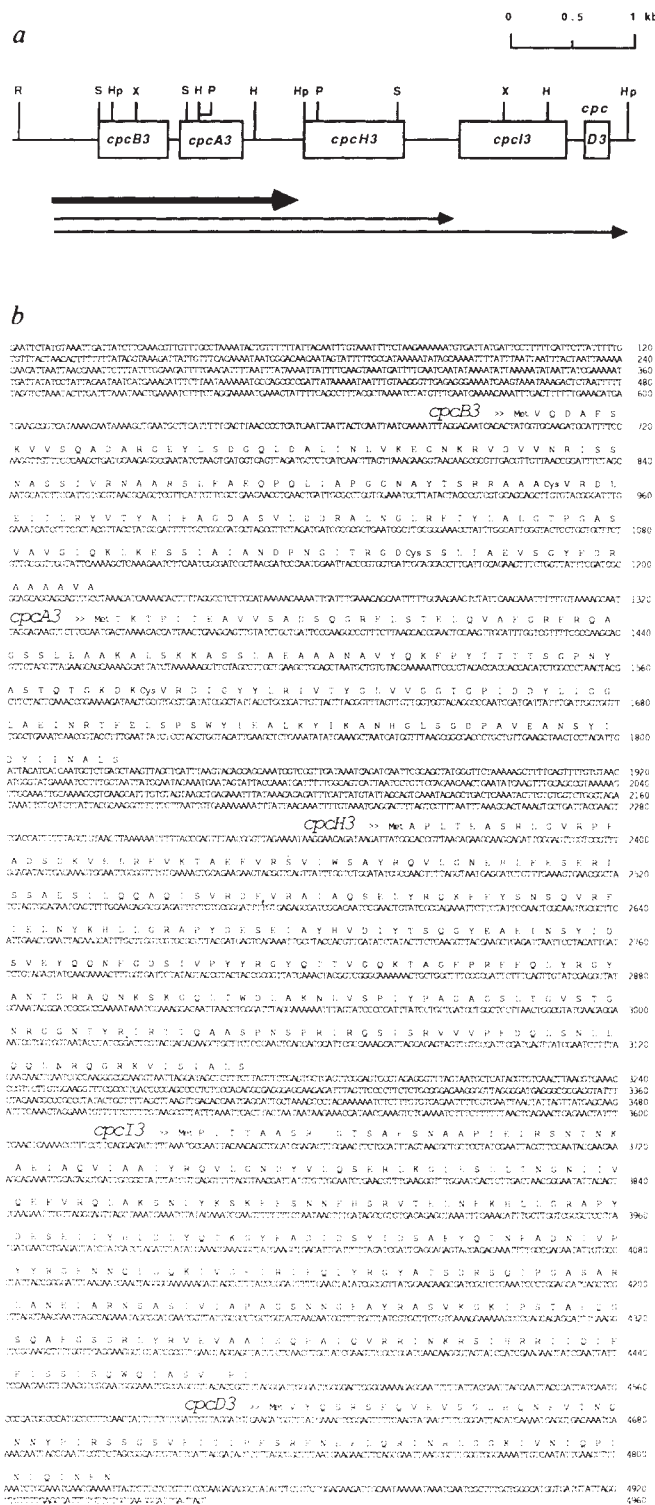


FIG. 1 Physical organization (a) and nucleotide sequence (b) of the *Calothrix* *cpc3* operon. a, Structural genes are indicated by open boxes and transcripts by arrows (see Fig. 3). Restriction sites are denoted as follows: H, *Hind*III; Hp, *Hpa*I; P, *Pst*I; R, *Eco*RI; S, *Sac*I; X, *Xba*I. b, Amino acids are denoted by the single letter code except for Met and Cys residues. METHODS. The molecular cloning of the 9.5-kb *Eco*RI fragment encompassing the *cpc3* locus, and the nucleotide sequencing of the *cpcB3* and *cpcA3* genes have been reported previously⁹. The remainder of the *cpc3* operon sequence was established similarly.

biochemically characterized, is transcribed at a very low level, regardless of the illumination spectrum⁹.

The sequence of the *cpc3* locus has now been completed (Fig. 1). It includes five open reading frames highly homologous to, and arranged in the same order as, the five genes of the *cpc2* operon⁸. These putative genes were therefore designated *cpcB3*, *cpcA3*, *cpcH3*, *cpcI3* and *cpcD3*. They are thought to encode the α - and β -subunits of a novel phycocyanin (hereafter designated PC3), and three linker polypeptides, respectively. These five genes, encoding over 1,000 amino acids, share a striking feature which distinguishes them from their counterparts in the operons *cpc1* and *cpc2*: they are free of codons specifying sulphur-containing amino acids, except for the five Met initiation codons and the three Cys codons corresponding to the phycobilin attachment sites (Fig. 2). This observation prompted us to test whether the *cpc3* operon, which remains silent in cells grown under standard conditions, is induced by sulphur deprivation.

Cells of *Calothrix* growing under white light in medium BG-11, containing 300 μ M sulphate¹⁰, were collected, washed, starved for 4 days in medium devoid of sulphate, and then inoculated in medium BG-11/S, containing 15 μ M sulphate. The brown cells turned green. Spectral analysis of whole-cell extracts revealed the disappearance of the PE absorption peak and a concomitant increase of the PC absorption peak (data not shown). The green cells could be indefinitely subcultured in BG-11/S medium. The change could be reversed by addition of sulphate to the deprived cultures or by subculturing them in fresh BG-11 medium. Messenger RNA hybridization with specific DNA probes showed that transcription of the operons encoding rod components was controlled by the availability of sulphur (Fig. 3). The *cpc3* operon was found to be highly expressed at low concentrations of sulphate in the culture medium. Three different mRNA species could be detected. The most abundant one, about 2-kilobases (kb), corresponded to the co-transcription of *cpcB3* and *cpcA3*, encoding PC3 α - and β -subunits. Two less abundant mRNAs were the 3.3-kb *cpcB3A3H3* and the 5-kb *cpcB3A3H3I3D3* transcripts. By contrast, the expression of the PC1-, PC2- and PE-encoding operons *cpc1*, *cpc2* and *cpe*, respectively, was switched off under conditions of sulphate limitation (Fig. 3). Transcription of the *apc* operon, encoding AP, persisted under these conditions (Fig. 3). Work is in progress to determine the mechanism of the transcription switch. There is no indication of a run of Met or Cys codons suggestive of a sulphur attenuator upstream of the *cpc3* genes.

The synthesis of sulphur-depleted phycobilisome proteins under conditions of sulphate limitation was further assessed by taking advantage of the fact that Met-free proteins, such as the putative *cpc3* gene product should resist cyanogen bromide (CNBr) treatment, whereas proteins encoded by *cpc1*, *cpc2*, *cpe* and *apc* should be cleaved into peptides. As shown in Fig. 4, purified phycobilisomes, or phycobiliproteins, from sulphate-unlimited and -limited cultures of *Calothrix*, which produce similar patterns in polyacrylamide-gradient gel electrophoresis, could indeed be distinguished by CNBr treatment.

That *cpc3*-encoded phycobilisome rods are functional is established by two main observations. Sulphate-limited *Calothrix* cells whose phycobilisomes lack PC1, PC2 and PE grow exponentially under white light, from which they must obtain their energy. Also, emission fluorescence experiments, performed on whole cells grown in sulphate-unlimited or -limited media, showed that light was in both cases efficiently transferred by phycobilisome-bound phycocyanin to the reaction centre of photosystem II (data not shown). In addition, the β -subunit of PC3 was found to bear a post-translationally γ -N-methylated Asn residue at position 72 (Dr A. N. Glazer, personal communication), as do other PC molecules¹¹.

The amino-acid sequences deduced from the *cpcA3* and *cpcB3* genes protrude by their initiation Met residues when they are aligned to their *cpc1* and *cpc2* counterparts, as if cleavage is required to put them into register (Fig. 2). Furthermore, the

FIG. 2 Pattern of sulphur occurrence in cyanobacterial light-harvesting proteins. Amino acids are denoted by the single-letter code. *a*, Phycocyanin α - and β -subunits^{8,21-25}. *b*, Phycocyanin-associated LPs^{8,23,25}. *c*, *Calothrix* 7601 phycoerythrin α - and β -subunits²⁶. *d*, Allophycocyanin α - and β -subunits (ref. 27). Alignments are compacted to show only positions among all the proteins that contain at least one Met (M) or Cys (C). Amino acids denoted by lower-case italics correspond to post-translationally matured sites, cleavable initiation Met¹² or chromophore-bearing Cys⁶. Initiation Met residues whose presence or absence at the N-terminus of mature proteins have not been experimentally proven are underlined. Sequences are listed in order of increasing number of Cys plus Met residues. Position number is indicated above the top sequence of each group. Species numbers correspond to those in the Pasteur Culture Collection of cyanobacteria. Species living in sulphur rich environments are indicated by a diamond, and *cpc3* gene products by an arrow. N-terminal microsequencing showed that PC1- or PC2 α - and β -subunits, PC3 α - and β -subunits, and AP α - and β -subunits start with M-K-T and M-L-D, T-K-T and V-Q-D, and S-I-V and A-Q-D, respectively. METHODS. N-terminal microsequencing was performed using an Applied

a	PHYCOCYANIN	α								β													
		-1	1	68	83	93	97	106		-1	1	30	38	44	79	82	86	109	134	143	153	157	
→	PC3 <i>Calothrix</i> 7601	m	T	T	c	I	G	I		m	V	L	V	I	A	c	L	A	L	N	c	I	
	<i>Anabaena</i> 7120	m	V	T	c	I	S	L		m	T	V	L	I	M	c	M	C	M	N	c	I	
	<i>Synechococcus</i> 6301	m	S	T	c	I	A	I		m	T	L	I	I	M	c	M	C	M	S	c	I	
	<i>Fischerella</i> 7603	m	V	T	c	I	S	L		m	A	V	L	I	M	c	M	C	M	N	c	I	
	PC1 <i>Calothrix</i> 7601	-	M	Q	c	M	C	L		-	M	L	M	I	M	c	M	C	M	G	c	M	
	PC2 <i>Calothrix</i> 7601	-	M	M	c	I	C	L		-	M	M	M	I	M	c	M	C	M	N	c	M	
♦	Plastid <i>C. caldarium</i>	-	M	T	c	M	C	M		-	M	M	L	I	M	c	M	C	M	N	c	M	
♦	<i>Synechococcus</i> 7002	-	M	T	c	M	C	M		-	M	V	S	M	M	c	M	C	M	M	c	Q	

b	LARGE LINKER	-1	49	167	260
→	<i>Calothrix</i> 7601 (<i>cpcH3</i>)	m	F	F	Q
→	<i>Calothrix</i> 7601 (<i>cpcI3</i>)	m	L	I	-
	<i>Anabaena</i> 7120	m	L	I	Q
	<i>Calothrix</i> 7601 (<i>cpcH2</i>)	m	M	M	R
	<i>Calothrix</i> 7601 (<i>cpcI2</i>)	m	M	M	L
♦	<i>Synechococcus</i> 7002	m	M	M	M

	SHORT LINKER	-1	1	42	46
→	<i>Calothrix</i> 7601 (<i>cpcD3</i>)	m	V	F	L
	<i>Calothrix</i> 7601 (<i>cpcD2</i>)	-	M	M	D
	<i>Anabaena</i> 7120	-	M	M	Y
♦	<i>Synechococcus</i> 7002	-	M	M	M

c	PHYCOERYTHRIN	α					β									
		1	59	82	139	144	1	48	49	57	59	71	77	80	107	132
	<i>Calothrix</i> 7601	M	C	c	c	M	M	c	M	M	c	C	M	c	C	M

d	ALLOPHYCOCYANIN	α										β										
		-1	1	6	76	80	84	114	132	137	139	140	159	-1	1	24	72	81	96	133	149	157
	<i>Calothrix</i> 7601	m	S	S	L	c	L	M	L	S	L	L	L	m	A	L	M	c	M	I	M	S
	<i>Anabaena</i> 7118	m	S	S	M	c	L	M	L	T	L	L	L	m	A	L	M	c	M	I	M	S
	<i>Synechococcus</i> 6301	m	S	S	M	c	L	M	L	T	L	L	L	-	M	L	M	c	M	I	M	S
	Plastid <i>C. paradoxa</i>	m	S	S	M	c	L	M	A	T	L	L	L	-	M	V	M	c	M	A	M	S
	<i>Fischerella</i> 7603	m	S	S	M	c	L	M	M	S	I	L	L	-	M	L	M	c	M	M	M	C
	<i>Anabaena</i> "cylindrica"	m	S	A	M	c	L	M	M	A	L	L	M	-	M	L	M	c	M	M	M	S
♦	Plastid <i>C. caldarium</i>	-	M	S	M	c	L	M	M	C	L	L	S	-	M	I	M	c	M	M	M	C
♦	<i>Calothrix</i> 7601 (<i>apcA2</i>)	m	S	M	M	c	M	M	M	T	M	L	L	-	M	M	M	c	M	M	M	C
♦	<i>Synechococcus</i> 7002	m	S	S	M	c	M	M	M	T	M	M	M	-	M	M	M	c	M	M	M	C

Biosystems model 470 A sequencer, on proteins transferred to Immobilon-P membranes after gel electrophoresis²⁸.

initiation Met residues of the five *cpc3* gene products are followed by residues known to be recognized by methionine aminopeptidase¹². Determination of the N-terminal sequences of purified PC α - and β -subunits from sulphate-limited and -unlimited cultures, showed that Met was indeed absent and present, respectively, at the beginning of the chains (see Fig. 2 legend). Both the α - and β -subunits of *Calothrix* AP, which are expressed regardless of sulphur availability (Fig. 3), have protruding initiation Met residues when their sequences are aligned to those of other phycobiliproteins (Fig. 2). Again, these residues were found to be cleaved post-translationally (see Fig. 2 legend). Conversely, both PE α - and β -subunits, which are silenced under conditions of sulphur limitation, begin with an unprocessed Met residue¹³. Note that PE has a high sulphur-containing amino-acid content compared to other *Calothrix* phycobiliproteins (Fig. 2c). It has been proposed that the susceptibility of proteins to degradation, and hence their cellular lifespan, is governed by the first aminoacid, and that initiation Met cleavage is crucial in this regard¹⁴. Our data show that sheer economy in Met or sulphur can also be important in the N-terminal maturation of proteins.

As is apparent from Fig. 2a, the eight known PC amino-acid sequences from six different cyanobacterial species fall into two clearcut classes that reflect the abundance of sulphur-containing amino-acids. *Calothrix* PC3 has the fewest sulphur-containing amino acids. Rod LPs closely follow the pattern of their corresponding PCs (Fig. 2b). The sulphur-containing amino acid distribution in the sequences of AP also matches that of PC but with less contrast (Fig. 2d). The consistent partition of PC and AP sequences into sulphur-rich and sulphur-depleted classes does not correspond to a phylogenetic dichotomy in the cyanobacterial lineage^{15,16}. It does, however, fit the available ecological data. Indeed, the two organisms with the PC and AP richest in sulphur, *Synechococcus* 7002 and *Cyanidium cal-*

darium, were isolated from habitats with a large excess of sulphate, sea water¹⁰ and acidic hot springs¹⁷, respectively. Conversely, organisms with sulphur-depleted PC and AP are found in low sulphate environments: *Calothrix* 7601, *Anabaena* 7120, *Cyanophora paradoxa* and *Synechococcus* 6301 in freshwaters¹⁰, and *Fischerella* 7603 in alkaline geothermal streams¹⁸. Phycobiliprotein sequences thus provide geochemical records of their evolutionary environments.

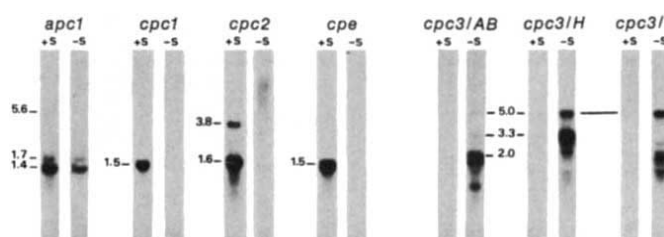


FIG. 3 Northern analysis of the mRNAs transcribed from *Calothrix* light-harvesting operons, that is, *apc* (AP), *cpc1* (PC1), *cpc2* (PC2), *cpe* (PE) and *cpc3* (PC3). Total RNA extracted from *Calothrix* cells grown in standard BG11 medium (+S) or sulphur limiting BG11/S medium (-S) (see text) were hybridized with: a 303 bp *DraI*-*HincII* fragment covering the 5' extremities of the *apc* transcripts (panel *apc*); a 1-kb *HindIII* fragment covering *cpcB1* and the 5' end of *cpcA1* (panel *cpc1*); an 836-bp *Clal*-*XbaI* fragment covering the *cpcB2* 3' end and the 5' end of *cpcA2* (panel *cpc2*); a 292-bp *HindIII* fragment covering the *cpcB3* 3' end and the 5' end of *cpeA* (panel *cpe*); a 463-bp *XbaI*-*HindIII* fragment covering the *cpcB3* 3' end and the *cpcA3* 5' end (panel *cpc3/AB*); an 859-bp *HindIII* fragment almost completely encompassing *cpcH3* (panel *cpc3/H*); and a 342-bp *XbaI*-*HindIII* fragment internal to *cpcI3* (panel *cpc3/I*). Transcript sizes are in kilobases. METHODS. Total RNA extractions were as previously described²⁶. Northern transfers and hybridizations were performed as in ref. 9.

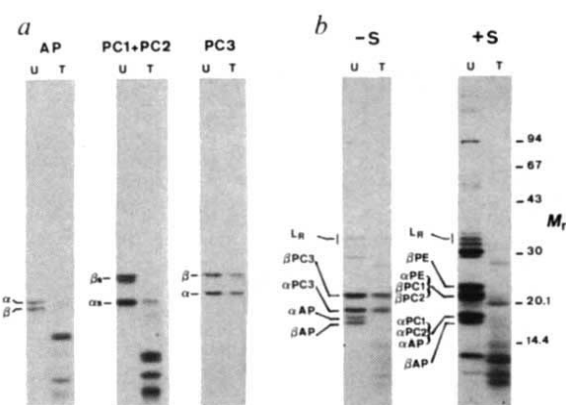


FIG. 4 Screening of Met-depleted phycobilisome proteins. U corresponds to crude samples and T to CNBr treated samples. *a*, Purified phycobiliproteins. *b*, Purified phycobilisomes from sulphate-limited (-S) and unlimited (+S) *Calothrix* cultures. L_r, rod linker polypeptides. *M_r*, relative molecular mass. METHODS. Purification of phycobiliproteins and phycobilisomes were carried out as previously described^{29,30}. The procedures for CNBr cleavage, and for electrophoresis and Coomassie blue staining of SDS 10–20% polyacrylamide gradient gels were as in refs. 31 and 30, respectively.

The constraints governing the folding, activity, specificity and stability of proteins are presumed to have shaped protein molecules during evolution¹⁹. Eradication of Met and Cys residues from phycobiliproteins indicates that economical constraints also came into play. That most microorganisms experience prolonged starvation in natural environments^{4,20} further suggests that the metabolic design of macromolecules is not an epiphenomenon, and raises two questions. First, to what degree could the elimination of an amino acid be extended in an organism? Second, are methionine and cysteine predisposed to disuse, or could this also be the fate of other amino acids? If so, changes in homologous proteins that seem functionally neutral may need to be reappraised in terms of the metabolic cost and availability of amino acids. □

Received 6 February; accepted 9 August 1989.

1. Roberts, R. B., Cowie, D. B., Abelson, P. H., Bolton, E. T. & Britten, R. V. *Studies of Biosynthesis in Escherichia coli* Publ. 607 (Carnegie Institute of Washington, Washington, D.C. 1955).
2. Pardee, A. B. *J. Biol. Chem.* **241**, 5886–5892 (1966).
3. Cuhel, R. L., Taylor, C. D. & Jannasch, H. W. *Arch. Mikrobiol.* **130**, 1–7 (1981).
4. Stanier, R. Y., Ingraham, J. L., Wheelis, M. L. & Painter, P. R. *The Microbial World*, 5th edn (Prentice-Hall, Englewood Cliffs 1986).
5. Tandeau de Marsac, N. *Bull. Inst. Pasteur* **81**, 201–254 (1983).
6. Glazer, A. N. *J. Biol. Chem.* **264**, 1–4 (1989).
7. Schirmer, T., Bode, W., Huber, R., Sidler, W. & Zuber, H. *J. molec. Biol.* **184**, 257–277 (1985).
8. Tandeau de Marsac, N. *et al. Photosyn. Res.* **18**, 99–132 (1988).
9. Mazel, D., Houmard, J. & Tandeau de Marsac, N. *Molec. Gen. Genet.* **211**, 296–304 (1988).
10. Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. & Stanier, R. Y. *J. gen. Microbiol.* **111**, 1–61 (1979).
11. Klotz, A. V. & Glazer, A. N. *J. Biol. Chem.* **262**, 17350–17355 (1987).
12. Tsunasawa, S., Stewart, J. W. & Sherman, F. *J. Biol. Chem.* **260**, 5382–5391 (1985).
13. Sidler, W., Kumpf, B., Rüdiger, W. & Zuber, H. *Biol. Chem. Hoppe-Seyler* **367**, 627–642 (1986).
14. Bachmair, A., Finley, D. & Varshavsky, A. *Science* **234**, 179–186 (1986).
15. Woese, C. R. *Microbiol. Rev.* **51**, 221–271 (1987).
16. Giovannoni, S. J. *et al. J. Bacteriol.* **170**, 3584–3592 (1988).
17. Allen, M. B. *Arch. Mikrobiol.* **32**, 270–277 (1959).
18. Binder, V. A., Locher, P. & Zuber, H. *Arch. Hydrobiol.* **70**, 541–555 (1972).
19. Creighton, T. E. *Proteins: Structures and Molecular Properties*. (Freeman, New York 1983).
20. Bachofen, R. *Experientia* **42**, 1179–1181 (1981).
21. Frank, G., Sidler, W., Widmer, H. & Zuber, H. *Hoppe-Seyler's Z. physiol. Chem.* **359**, 1491–1507 (1978).
22. Glazer, A. N. *Biochim. biophys. Acta* **768**, 29–51 (1984).
23. Belknap, W. R. & Haselkorn, R. *EMBO J.* **6**, 871–884 (1987).
24. Offner, G. D. & Troxler, R. F. *J. Biol. Chem.* **258**, 9931–9940 (1983).
25. de Lorimier, R. *et al. Proc. natl. Acad. Sci. USA* **81**, 7946–7950 (1984).
26. Mazel, D. *et al. Nucleic Acids Res.* **14**, 8279–8290 (1986).
27. Houmard, J., Capuano, V., Coursin, T. & Tandeau de Marsac, N. *J. Bacteriol.* **170**, 5512–5521 (1988).
28. Matsudaira, P. *J. Biol. Chem.* **262**, 10035–10038 (1987).
29. Guglielmi, G. & Cohen-Bazire, G. *Protistologica* **20**, 393–413 (1984).
30. Tandeau de Marsac, N. & Houmard, J. *Meth. Enzym.* **167**, 318–328 (1988).
31. Gross, E. *Meth. Enzym.* **11**, 251–253 (1967).

ACKNOWLEDGEMENTS. We are grateful to Dr N. Tandeau de Marsac for her constant support. We also thank Drs J. Houmard, G. Cohen, W. Saurin, T. Damerval and G. Stanier for comments; Drs H. Bedouelle, C. J. Thompson, O. Bärzu, F. Rouyer, P. Sonigo, I. Old, R. Ford and E. J. Johnson for comments on the manuscript; Drs R. Rippka, G. Guglielmi, J. C. Thomas, K. Elmorjani, C. Carles for technical help; Dr A. N. Glazer for providing unpublished results; and L. Girardot for secretarial assistance.

Fitness reduction associated with the deletion of a satellite DNA array

Chung-I Wu, John R. True & Norman Johnson

Department of Biology, University of Rochester, Rochester, New York 14627, USA

SATELLITE DNA refers to a class of tandem repeats of very simple sequences, usually A+T or G+C rich, which form a satellite band on a CsCl gradient. Their ubiquity and abundance in higher eukaryotes have led to speculation about their functions^{1–3}. It has often been suggested that satellite DNAs are merely innocuous genetic parasites or comprise 'junk' DNA^{2–4}. The recent identification of an array of satellite DNA repeats as the *Responder* (*Rsp*) locus of *Drosophila melanogaster* provides a new perspective on these elements⁵. *Rsp* is in the centromeric heterochromatin of most natural second chromosomes⁶. It causes spermatids bearing it to degenerate after meiosis when the homologous second chromosome is a *Segregation Distorter* (*SD*) chromosome. That is, *SD* targets the *Rsp* locus on its homologue for destruction during spermatogenesis, causing meiotic drive. Why then does the *Rsp* locus, a large array of satellite repeats, exist at all? One plausible explanation is that its existence contributes to the fitness of flies bearing it, compensating for the loss through meiotic drive. A direct demonstration of the usefulness of any family of satellite DNA is to compare the fitnesses of individuals with and without it. Previously, such an experiment has been difficult because the absence of a characteristic phenotype has precluded an efficient selection of deletion mutations. In this report we attempt to demonstrate a fitness reduction associated with the deletion of *Rsp* satellite DNA as well as the life stages at which such a reduction occurs.

The *Rsp* locus has been deleted from chromosomes and some deletion homozygotes found to be viable and fully fertile⁶. Deletion chromosomes no longer respond to the distorting action of *SD*, indicating the neomorphic nature of *Rsp*. In our experiments, we measured fitness differences between a deletion chromosome, called R16 (*cinnabar brown*^{R16}), and the chromosome from which it was derived, called CB (*cinnabar brown*)⁶. These two chromosomes differ in the amount of *Rsp* satellite repeats that they carry (20 and 700 copies, respectively⁵) but are otherwise genetically identical. Cytological examination indicates that the deletion on R16 is small and is coextensive with the *Rsp* region⁷. Because any *SD* chromosome would induce degeneracy of spermatids bearing the *Rsp* locus, *SD*/CB males transmit only 1% of CB-bearing sperm whereas *SD*/R16 males transmit 50% of R16 bearing ones⁶.

Our first experiment demonstrated that *Rsp* satellite DNA can be a liability to chromosomes bearing it when the population also contains *SD* chromosomes, as most natural populations do. Figure 1 shows that the frequency of R16/R16 individuals, relative to the individuals bearing CB, rose sharply from 0.06 to 0.41 in only five generations. The observation is as expected, because *SD* chromosomes should prevent the transmission of CB chromosomes but not of R16 chromosomes in heterozygous males. The identification of genotypes was aided by a quick DNA hybridization procedure that permitted scoring several hundred flies at once. Squash blots of R16/R16 flies (top row of the inset, Fig. 1) indeed failed to hybridize detectably to the *Rsp* probe, which hybridized strongly to squash blots of CB/R16 flies (bottom row).

If such deletions are so advantageous in the presence of *SD* chromosomes why in nature don't these deletion chromosomes replace chromosomes bearing *Rsp*? The frequency of *SD* is ~3% in most populations⁸. Such a worldwide distribution should be sufficient to cause deletions of *Rsp* to become fixed

in natural populations^{8,9}. Previous surveys, however, showed that chromosomes deficient in *Rsp* are relatively uncommon^{10,11}. A logical deduction is that *Rsp* satellite DNA has a fitness advantage that compensates for the loss in fitness incurred through meiotic drive. In our second experiment, we let CB chromosomes compete with R16 chromosomes in the absence of *SD* chromosomes. Because selection against the R16 deletion, if present at all, was expected to be weak, long-term population cages were set up. To counter an increase in gene frequency variance due to genetic drift, we maintained large populations throughout the course of the experiment.

Figure 2a,b shows the changes in the frequency of R16/R16 individuals in two cages; with time R16 flies steadily decreased in frequency in both cages. Note that the time scale in these figures is five times that in Fig. 1. There were five measurements from cage 1 and four measurements from cage 2. The frequency of R16/R16 flies decreased between all successive measurements ($P = 0.57 < 0.01$). Curve fittings of observed changes to theoretical trajectories based on the standard selection formula¹² yielded a selection coefficient of 0.008 and 0.012 per day for the two cages, respectively. The experiment, therefore, demonstrates the fitness contribution of *Rsp* satellite DNA to be about 13–19% per generation. Dominance in this range of gene frequency

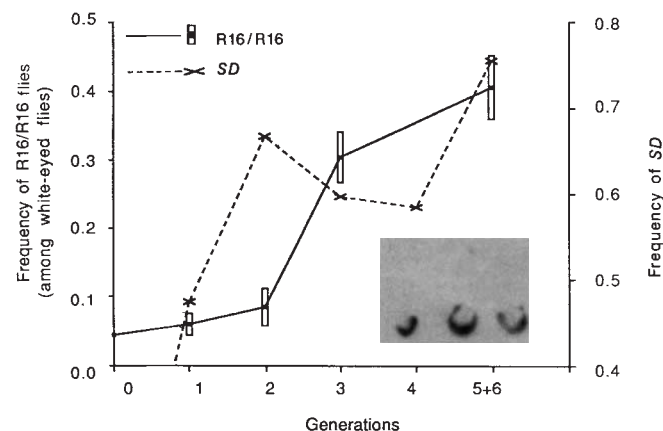


FIG. 1 Changes in the frequency of R16/R16 flies among white-eyed flies (CB/CB, CB/R16 and R16/R16) in the presence of *SD*.

METHODS. Both CB and R16 carry *cinnabar* (*cn*) and *brown* (*bw*) recessive mutations, and homozygotes of double mutations are white eyed. The *SD* chromosome used is *SD_{MAD}*, which carries inversions that suppress recombination between the *Rsp* locus, *cn* and *bw*. During the experiment, recombination between *cn* and *bw* was not observed. The experiment was started with 250 pairs of flies with the following composition: *SD/SD* (4.7%), *SD/CB* (13.8%), *CB/CB* (68.7%), *CB/R16* (8.4%) and *R16/R16* (4.4%), close to the Hardy-Weinberg ratio. The last three types are white eyed. The experiment was of a discrete-generation design conducted in a big cage at 25 °C. In each generation, adults were allowed to lay eggs on fresh food for 6–8 days and then sorted according to eye colour and sex. White-eyed flies were stored at –70 °C for genotype identification by squash-blotting. The bottles containing larvae were left in the cage for the emergence of the next generation. Newly emerged flies were allowed to mate without any handling. Six new bottles were put in to start the next generation two days after the first adult emerged. The change in the frequency of the *SD* chromosome was reflected in the proportion of red-eyed flies. The more *SD* chromosomes present, the greater the disadvantage of CB relative to R16. Whereas R16 had been steadily replacing CB, the largest increase took place between generations three and four, immediately after the largest increase in *SD* in the previous generation. The frequency of *SD* is given as $1 - w^{0.5}$, where w is the proportion of white-eyed flies in the total. Genotypes of white-eyed flies were determined by squash-blotting adult flies on nitrocellulose paper, which was then soaked in 2% SDS before being placed on denaturing and neutralizing buffer. Hybridization was in the presence of the *Rsp* satellite DNA probe. The results are shown in the inset. Top row, R16/R16 flies; bottom row, R16/CB flies.

(0.35–0.61) does not change the theoretical trajectory appreciably although the best fit on data from either cage is obtained when the CB chromosome is assumed to be dominant. We have therefore studied the fitness differences between CB/R16 individuals and R16/R16 individuals in greater detail.

At what life stages does the *Rsp* satellite DNA exert its fitness effect? We thought that the stage of male fertility was the most probable for several reasons. First, the 'negative phenotype' of *Rsp* satellite DNA, namely *Segregation Distortion*, is manifested during spermatogenesis. Second, the male fertility component of fitness is important in *Drosophila*, especially in meiotic drive systems¹³. Third, it has been proposed that satellite DNAs function primarily in germ cells². Table 1 presents some surprising results. Males homozygous for R16, with little *Rsp* satellite DNA, were as fertile as CB/R16 males. Because *Drosophila*

TABLE 1 Measurement of fitness components

Fertility			
	Males	Females	
CB/R16	98.0 ± 4.86 (21)	72.3 ± 3.35 (28)	
R16/R16	103.1 ± 6.57 (17)	63.0 ± 6.44 (11)	
Viability of R16/R16 relative to CB/R16			
	Males	Females	Total
Cross I	—	—	0.650 ± 0.078 (297)
Cross II	0.564 ± 0.087 (183)	0.681 ± 0.099 (195)	0.622 ± 0.066 (378)
Developmental time			
	Proportion of R16/R16 flies that emerged on: day 10	day 11	days 12–15
	0.201 ± 0.034 (139)	0.200 ± 0.037 (120)	0.145 ± 0.042 (69)

Numbers in parentheses are sample sizes. Virgin females are referred to as females. Flies used for the fertility tests were the F_2 resulting from the following crosses: CB/CB females × R16/R16 males (P); R16/R16 females × CB/R16 males from P (F_1). The F_2 individuals were either CB/R16 or R16/R16. Identification of genotypes was done after the fertility tests were completed by squash-blotting. The scheme ensured an identical culture and genetic background, which was identical between the two parental stocks. Male fertility: Each freshly emerged male was mated to three CB/CB females (4 days old) for three days and then transferred to a new vial containing three more females for three more days. The first trio were also transferred to new yeast medium for further egg-laying. Progeny from all three vials were counted and added. Each male was thus mated to six females with each female allowed a six-day egg-laying period. Because a fully inseminated CB/CB female in a six-day period can produce more than 50 progeny, the male fertility test was carried out under a sperm-limiting condition. Female fertility: Each female was 2-days old before being given three CB/CB males (4-days old) and allowed to lay eggs for 8 days in the presence of the males. Egg production is the limiting factor in the female fertility test. Viabilities: random sample of F_2 individuals from each of the two crosses were squash-blotted. Cross I: R16/R16 females × CB/CB males (P); R16/R16 females × CB/R16 males from P (F_1). Cross II was identical except that the P generation was CB/CB females × R16/R16 males. The ratio of the two genotypes in F_2 CB/R16 and R16/R16, was expected to be 1 in the absence of any viability difference. An unbiased estimator of the relative viability (v) and its variance are used. [$v = x/(y+1)$ and $\sigma^2 = v(1+v)^2/(x+y)$, where x is the number of R16/R16 flies and y is the number of CB/R16 flies²⁷.] In Cross I, the sex of F_2 individuals was not distinguished. Developmental time: well-yeasted food bottles were placed into Cage I (Fig. 2) for 4 h to collect eggs. The bottles were then cleared of adults, capped (to prevent further egg laying) and returned to the cage until the emergence of adults 10 days later. The proportion of R16/R16 flies in each day's sample was then determined.

males usually ejaculate more sperm than females can utilize¹⁴, males suffering some extent of spermatogenic failure may have appeared as fertile as normal males if the experiment had not been done under sperm-exhaustion conditions¹³⁻¹⁵. To ensure the detection of spermatogenic differences, very young males can be used^{14,15}, or many females supplied, or pre-inseminated females can be mated for detecting sperm displacement¹³. We used a combination of the first two tactics. The total productivity was limited by the supply of sperm because females under the same condition would have been much more fecund if more males were available (see Fig. 2 legend). The absence of an observable difference in male fertility suggests that *Rsp* satellite DNA has little or no role during normal spermatogenesis and, whatever the normal function of *Rsp* satellite DNA, it is distinct from the event of segregation distortion. The fertility difference in females is statistically insignificant ($P > 0.1$).

Despite the lack of observable fertility differences due to the presence of *Rsp* satellite DNA, large differences in viability could be detected, as shown in Table 1. The experiment, repeated with the reciprocal cross, yielded nearly identical results. The viability effect seemed to be independent of sex. The large difference in viability was higher than the measurement taken from the population cages, where total selection per generation is estimated to be ~ 0.15 . The discrepancy was due primarily to the measurement of individual fitness components (viability and fertility) under more severe conditions than in the long-term cages. Fertility was measured under sperm and egg exhaustion conditions, and egg-to-adult viability for all genotypes was appreciably lower than that in the optimal cultures. The important point is that no fertility differences could be detected whereas large viability differences were observable under stringent conditions. Finally, we measured the relative developmental time by counting the proportion of R16/R16 individuals among the total in the sequential samples. The proportion remained nearly constant during the first two days. During the

subsequent four days, the proportion of R16/R16 individuals actually decreased although this decrease is statistically insignificant and can be best attributed to a small sample size. Within the resolution of our measurement, we conclude that R16/R16 flies do not develop more slowly than the other two types.

There are several explanations for the observed fitness deficiency of the R16 deletion chromosome, relative to the original CB chromosome. We can rule out deleterious mutations in the background (X, Y or the third chromosomes) because CB and R16 stocks had been mixed for five generations before the first measurement was taken (see fig. legends). The continual decline of R16 chromosomes in the ensuing nine months also supports this point. There is a formal possibility that the reduced fitness of R16 was due to a deleterious mutation of R16. Such a mutation would have had to be sufficiently close to *Rsp* to be consistent with the steady decline of R16 but should have been outside of the R16 deletion for two reasons. First, Pimpinelli and Dimitri⁷ have shown cytologically that the deletion does not extend beyond the region occupied by the *Rsp* repeats. The R16 deletion is indeed much smaller than heterochromatic deletions or duplications used in previous studies^{2,16} and genes in 2R heterochromatin are all mapped some distance away from this deletion^{7,17}. Second, there is no evidence for single-copy sequences within the region of *Rsp* repeats. From a lambda library and a cosmid library, we have obtained many different clones in the *Rsp* region. These clones showed only dispersed repetitive sequences adjacent to *Rsp* repeats. The interspersed repetitive sequences within the R16 deletion are not likely to account for the observed fitness differences of R16 because there are many more of these dispersed sequences outside of the R16 deletion than within it. Our observations, therefore, are best explained by the deletion of *Rsp* repeats although we cannot completely rule out the existence of an unidentified, but closely-linked secondary lesion, induced by the same irradiation that deleted

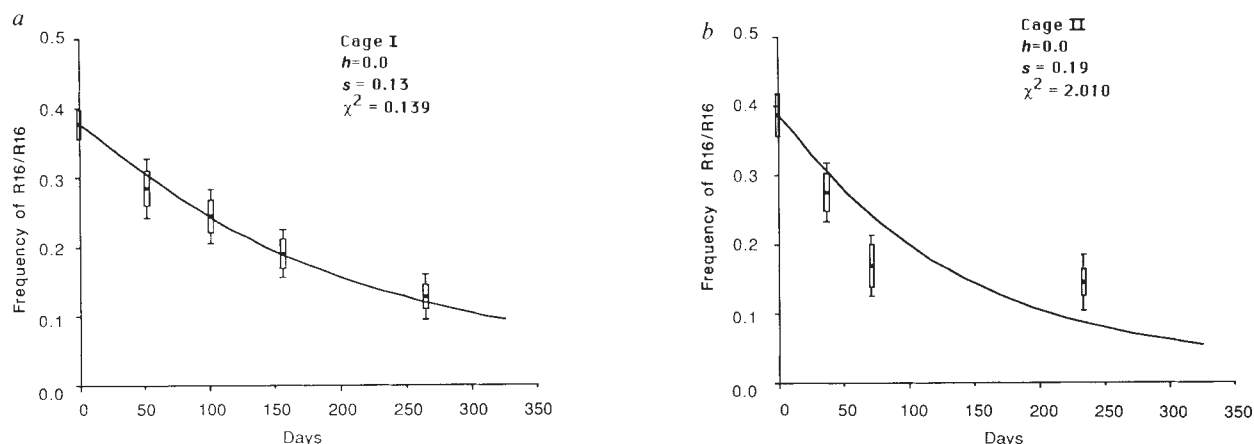


FIG. 2 *a, b*, Changes in the frequency of R16/R16 flies in the absence of *SD*. METHODS. Each cage was started with ~ 300 pairs of flies, 40% R16/R16 and 60% CB/CB. The populations were then allowed to expand to 3,000–5,000 flies. The first sampling, day 0 in the figure, was done 8-weeks later. Although the two stocks were genetically identical when constructed⁷, such a shuffling period allowed further randomization of the genetic background. The populations were maintained at 25 °C with overlapping generations, and the population sizes never dropped below 3,000. Sampling were done every 50 days or so by collecting all flies that emerged from several food bottles. All samples consisted of 200–400 flies, squashed-blotted for genotype determination. The binomial sampling standard errors are indicated by the thick bar. The stochastic standard errors due to genetic drift are represented by the thin bar. Stochastic variances are calculated by $V_t = p(1-p)(1 - e^{-t/2N})$ (ref. 12), where p is the frequency of R16, t is the number of generations between two successive data points and N is the effective

population size. N is assumed to be 2,000 ($\sim 70\%$ of the minimum of the actual number). A least-square fitting of observations to theoretical calculations was carried out. Theoretical values were obtained using the formula, $\Delta q = -spq[q + h(p-q)/(1-2pqhs - q^2s)]$, where s is the coefficient of selection against R16, h is the degree of dominance, q is the frequency of R16, and $p=1-q$. A series of s and h values were used to find the combination that minimized χ^2 , (observed – expected)²/expected, summed over all data points. The expected frequency of R16/R16 in each generation was calculated as $q^2(1-s)/(1-2pqhs - q^2s)$. The calculation assumes only pre-adult selection, which is supported by the data of Table 1. The generation time, the average age of mothers of sampled offspring, was assumed to be 16 days. (The mean emergence time was 11 days and we assumed the generation time was five days after emergence.) The best fit is given in the figure.

Rsp repeats. It is worth noting that there is a paucity of genes in centromeric heterochromatin.

The prevailing view that satellite DNA is mostly 'junk'^{2-4,18}, whose presence or absence has no bearing on the fitness of its carriers, has been widely accepted. Most of the support for this came from interspecific comparisons^{2,18}. By adding extra heterochromatic materials or by deleting nearby essential (ribosomal RNA) genes, previous studies^{2,16} only addressed the issue indirectly. We have provided the first direct test of this hypothesis by comparing the fitnesses of *Drosophila* with, and without, a well characterized array of satellite repeats. A fitness effect is clearly detectable. The observed effect is also inconsistent with the view that the functions of satellite DNA, if any, must be in the germ cells².

It is premature to generalize our conclusion to other satellite DNAs. After all, *Rsp* was identified because of segregation distortion. Nevertheless, our results show that this negative phenotype (in germ cells) can be uncoupled from its positive

functions (in the soma). It is possible that other satellite DNAs perform similar positive functions without the dramatic negative phenotype associated with *Rsp*. *Rsp* satellite DNA has a marked sequence-directed curvature (that is, DNA bending; unpublished results), which is often associated with A+T-rich DNAs such as mouse satellite DNA¹⁹. Bent DNA is important in nucleosome assembly²⁰ and chromatin condensation¹⁹—it should be noted that segregation distortion results from aberrant chromatin condensation in spermatids bearing *Rsp*²¹. Parallel systems involving heterochromatic materials, such as Sex-Ratio distortion²² or B chromosome segregation²³ may be a logical next step for investigation. Attempts to define the genetic roles of heterochromatic materials, such as Position Effect Variegation²⁴, and to identify satellite-DNA-specific binding proteins^{25,26} are clearly promising approaches. It is far fetched to think all satellite DNAs have a useful role, but it is equally unwise to label them universally as junk in the absence of any other direct proof. □

Received 15 May; accepted 10 August 1989.

1. Brutlag, D. L. *A. Rev. Genet.* **14**, 121-144 (1980).
2. Miklos, G. L. G. in *Molecular Evolutionary Genetics* (ed. McIntyre, R. J.) 241-322 (Plenum, New York, 1985).
3. Ohno, S. in *Evolution of Genetic Systems* (ed. H. H. Smith) 366-370. (Gordon and Breach, New York, 1970).
4. Smith, G. P. *Science* **191**, 528-535 (1976).
5. Wu, C.-I., Lyttle, T. W., Wu, M. L. & Lin, G. F. *Cell* **54**, 179-189 (1988).
6. Ganetzky, B. *Genetics* **86**, 321-355 (1977).
7. Pimpinelli, S. & Dimitri, P. *Genetics* **121**, 765-772 (1989).
8. Crow, J. F. *Scient. Am.* **240**, 134-146 (1979).
9. Charlesworth, B. & Hartl, D. L. *Genetics* **89**, 171-192 (1978).
10. Hiraizumi, Y. & Thomas, A. M. *Genetics* **106**, 279-292 (1984).
11. Temin, R. G. & Marthas, M. *Genetics* **107**, 375-393 (1984).
12. Crow, J. F. & Kimura, M. *An Introduction to Population Genetics Theory* (Burgess, Minneapolis, 1970).
13. Wu, C.-I. *Genetics* **105**(3), 651-662 (1983).
14. Lefevre, G. & Jonsson, U. B. *Genetics* **47**, 1719-1736 (1962).

15. Hartl, D. L., Sandler, L. & Crow, J. F. *Proc. natn. Acad. Sci. U.S.A.* **58**, 2240-2245 (1968).
16. Yamamoto, M. & Miklos, G. L. C. *Chromosoma* **66**, 71-98 (1978).
17. Brittnacher, J. G. & Ganetzky, B. *Genetics* **121**, 739-750 (1989).
18. John, B. & Miklos, G. *The Eukaryote Genome in Development and Evolution*. (Allen & Unwin, London, 1989).
19. Radic, M. A., Lundgren, K. & Harkkalo, B. A. *Cell* **50**, 1101-1108 (1988).
20. Hsieh, C. H. & Griffith, J. D. *Cell* **52**, 535-544 (1988).
21. Tokuyasu, K. T., Peacock, W. J. & Hardy, R. W. Z. *Zellforsch. Mikrosk. Anat.* **124**, 479-506 (1972).
22. Wu, C.-I. & Beckenbach, A. T. *Genetics* **105**, 71-86 (1983).
23. Nur, U., Werren, J. H., Eickbush, D., Burke, W. & Eickbush, T. *Science* **290**, 512-514 (1988).
24. Dorn, R., Heymann, S., Lindigkei, R. & Reuter, G. *Chromosoma* **93**, 398-403 (1986).
25. James, T. C. & Elgin, S. C. R. *Molec. cell. Biol.* **6**, 3862-3872 (1986).
26. Strauss, F. & Varshavsky, A. *Cell* **37**, 889-901 (1984).
27. Haldane, J. B. S. *J. Genet.* **54**, 294-296 (1956).

ACKNOWLEDGEMENTS. We thank Scott Erdman for helping set up the cages, Barry Ganetzky for providing the R16 stock, Mao-Lien Wu for technical assistance, Uzi Nur and Adina Merenlender for comments, and Brian Charlesworth and the other reviewer for many constructive criticisms. The study was supported by an NIH grant to C.-I. W.

Functional replacement of a protein-induced bend in a DNA recombination site

Steven D. Goodman & Howard A. Nash

Laboratory of Molecular Biology, National Institute of Mental Health, Bethesda, Maryland 20892, USA

IN recent years the capacity of proteins to bend DNA by binding to specific sites has become a widely appreciated phenomenon¹⁻⁷. In many cases, the protein-DNA interaction is known to be functionally significant because destruction of the DNA site or the protein itself results in an altered phenotype. An important question to be answered in these cases is whether bending of DNA is important *per se* or is merely a consequence of the way a particular protein binds to DNA. Here we report direct evidence from the bacteriophage lambda integration system that a bend introduced by a protein is intrinsically important. We find that a binding site for a specific recombination protein known to bend DNA can be successfully replaced by two other modules that also bend DNA; related modules that fail to bend DNA are ineffective.

Bacteriophage λ requires two DNA binding proteins, Int and integration host factor (IHF), to integrate site-specifically into the *Escherichia coli* chromosome⁸. Although it is known that Int, a phage-encoded protein, performs the actual DNA-strand exchanges, the function of the *E. coli* protein, IHF, is less well understood. IHF is a heterodimeric protein with a relative molecular mass of ~20K which binds specifically to three sites within the phage λ recombination locus, *attP*. Mutational analy-

sis of these sites indicate that all three are required for efficient recombination⁹ and IHF is known to bend DNA on binding to each of its sites within *attP* (refs 10,11). Many experiments indicate that the active form of *attP* is a condensed nucleoprotein structure, the intasome, in which Int protomers, bound at sites 100-200 base pairs (bp) apart, interact with one another¹². Experiments in our laboratory have shown that IHF is necessary for intasome formation¹³. IHF could assist the intasome by participating in a network of protein-protein interactions with other protomers of IHF and/or with Int. Another possibility is that, by changing the shape of DNA, IHF could facilitate the interaction of other components of the intasome. In this case, it is the conformation of DNA produced by IHF that is critical for efficient recombination and not the protein itself. We have now tested whether bending is sufficient for IHF function in recombination.

We reasoned that if changing the shape of DNA was the primary function of IHF, we should be able to replace the protein by unrelated elements that also bend DNA. Our initial approach has been to focus on just one of the three IHF binding sites within *attP*, the central or H2 site. The cyclic AMP receptor protein (CRP) of *E. coli* was chosen for the replacement because it is apparently unrelated to IHF in amino-acid sequence and structure but, like IHF (refs 11, 14), is believed to bend DNA sharply, by more than 90° (ref. 15). Our approach in replacing an IHF site of *attP* with a CRP site took into account the expectation that the precise parameters of the two protein-induced bends are not identical. We hoped that inserts of different lengths and orientations might compensate for such differences as well as differences in the size and shape of IHF as compared with CRP. Our strategy for incorporating the CRP binding site into *attP* is shown in Fig. 1. A 38 bp fragment

containing the *lac* CRP site was cloned between tandemly repeated polylinkers. Restriction-endonuclease digestion with different pairs of enzymes created a collection of fragments, all of which had the same CRP site but differed from each other by variable lengths of spacer DNA on either end. These fragments were ligated into a gap in *attP* created by the deletion of the H2 site. Individual chimaeric *attP* sequences (*attP*-CRP) were assayed *in vitro* for integrative recombination with purified IHF and Int in the absence or presence of CRP (gift from J. Harman). Table 1 summarizes our results. In the absence of CRP, no *attP*-CRP chimaera recombines better than the control plasmid with the H2 site destroyed by base substitution⁹. In the presence of CRP, however, several chimaeras yield significant recombination and one, pHN1030, functions almost half as well as a wild-type *attP*. Since all chimaeras contain a CRP site, differences in efficiency probably reflect requirements for inserts with proper spacing and/or helical phasing. This can be seen most clearly when pairs that differ by a 4 bp alteration at a restriction site (for example pHN1030 and pHN1011) are compared. Several additional experiments (not shown) indicate that the chimaeras, as typified by pHN 1030, resemble wild-type *attP* in recombination properties. First, the products of recombination with chimaeras have the expected restriction map. Second, the time course of recombination with chimaeras parallels that of *attP*, being approximately linear under our conditions. Third, recombination of the chimaeras depends strongly on supercoiling, as does that of *attP*. It should also be noted that CRP fails to stimulate recombination of pHN 1030 in the absence of cAMP.

TABLE 1 Recombination of *attP*-CRP plasmids

<i>attP</i> plasmid No.	Insert size* (bp)	Orienta- tion†	Distance from arm to bend centre‡	Distance from core to bend centre§	Recombination (% wild type) -CRP +CRP
pHN986	74	NA	NA	NA	(100) 102
pHN1006	74	NA	NA	NA	5.9 4.9
pHN1030	126	—	74 bp	52 bp	2.5 41
pHN1031	103	+	52 bp	51 bp	2.4 30
pHN1014	109	+	69 bp	40 bp	1.2 15
pHN1010	88	+	48 bp	40 bp	0.9 11
pHN1008	146	+	64 bp	82 bp	0.6 11
pHN1011	122	—	74 bp	48 bp	1.3 9
pHN1013	99	+	48 bp	51 bp	1.2 6.7
pHN1007	87	—	51 bp	36 bp	<0.1 0.6
pHN1009	94	+	36 bp	58 bp	<0.1 <0.1
pHN1012	94	—	46 bp	48 bp	<0.1 <0.1

Plasmids above the dotted line are controls. pHN986 is wild type, pHN1006 contains a 4 base change introduced into the H2 site which inactivates IHF binding⁹. Plasmids below the dotted line are *attP*-CRP chimaeras. Recombination reactions were performed as stated in Fig. 2 except that cAMP (25 μ M) was added to each reaction and CRP (250–500 nM) was added where indicated. pHN986 was tested 22 times; the mean value for recombination in the absence of CRP was 40% (with a range of 11–67%). All other data on the extent of recombination were expressed relative to this value. In 14 tests, the range of relative recombination values for plasmid pHN1006 was 0.2–18% in the absence of CRP and 2–16% in the presence of CRP. For the plasmid pHN1030 (24 tests) the ranges were 0.1–5.5% and 14–85% in the absence and presence of CRP respectively. NA, not applicable.

* The size of the inserted fragment at the original *NdeI* and *SphI* site of pHN986.

† An arbitrary convention to establish the orientation of the CRP site. + signifies the strand listed for CRP in the legend of Fig. 1 is contiguous with the 'top' strand of *attP*; —, is the opposite orientation.

‡ The number of base pairs from the putative bend centre¹⁶ in the insert to the *NdeI* site.

§, The number of base pairs from the putative bend centre in the insert to the *SphI* site.

Although the success of the replacement of IHF at H2 by CRP argues strongly for the role of DNA bending as the critical function of IHF, we cannot rule out the possibility that CRP also participates in a network of protein-protein interactions. We think this is unlikely because CRP is apparently unrelated to IHF in primary sequence and overall structure. Nevertheless, to eliminate this possibility more conclusively we have extended our 'bend swap' experiments by replacing the H2 site with DNA that does not depend on protein to create a bend. For this we used a segment of DNA that contains 6 repeats of a tract of 6 adenines phased so as to form a stable bend of $\sim 120^\circ$ (sequence A₆-1/1; ref. 16, gift from S. Zinkel). We repeated the cloning protocol of Fig. 1 using the 'A tract' DNA instead of the CRP binding site. The 'A tract' was liberated from the vector by cutting within the polylinker as before, and a population of fragments containing a stable bend was then cloned into plasmid pHN986 in place of the H2 locus. The collection of chimaeras (*attP*-K) were then used as substrates for *in vitro* recombination. The results of a representative group of reactions can be seen in Fig. 2. As was observed with the *attP*-CRP chimaeras, there is a wide range in the amount of recombination observed with *attP*-K plasmids. One chimaera (pHN1045, lane 4) recombines at 50–60% of the level of wild-type *attP*. Addition of 4 bp to this chimaera greatly decreases its efficiency (pHN1050, lane 9) as does recloning of the insert in the opposite orientation (pHN1052, lane 11). This shows that in addition to the presence of the sequence-directed bend, spacing and/or phasing of the bend is critical. The importance of the bend is highlighted by our construction of a chimaera that has an insert (the *TaqI* 44667–44809 fragment of λ) of precisely the same length and base composition as the insert in plasmid pHN1045 but which has DNA of normal electrophoretic mobility on a native polyacrylamide gel; this chimaera (pHN1053) produces few or no *in vitro* recombinants (see Table 2). In addition, just as with *attP*-CRPs, *attP*-Ks have an approximately linear rate of recombination and a strong requirement for supercoiling (data not shown). We also asked if the *attP*-K chimaeras were active during *in vivo* site-specific recombination. In a recent study in which the CRP binding site of the *gal* promoter was replaced by a sequence directed bend, a marked discrepancy was noted between *in vivo* transcription and that in a purified *in vitro* system (H. Buc, personal communication.). However, for each *attP*-K tested (Table 2), the amount of *in vivo* recombination relative to wild type *attP* agrees with that expected from its *in vitro* performance.

We have now successfully employed three types of sequences at the H2 locus; the natural IHF binding site, 'A-tract' DNA and a CRP binding site. The pattern of our success allows two conclusions to be drawn. First, the fact that the segments have very different lengths demonstrates that *attP* has a surprising tolerance for spacing between its component parts. Second, the fact that the segments are unrelated except for adopting a bent conformation provides compelling evidence that the role of IHF at H2 is merely to bend DNA and that IHF function at H2 does not depend on protein-protein interactions. We have performed two additional experiments that support this interpretation. First, we assayed for IHF binding to the inserts used in the chimaeras. Because these inserts did not contain an IHF consensus sequence¹⁷, we did not expect binding but we wondered if bending by CRP or the 'A-tract' would relieve IHF of its sequence specificity. If this were the case, our conclusion would be unfounded. To test for IHF binding we incubated the inserts (~ 300 bp fragments of pBend-CRP or pHN1036) with IHF and/or CRP, and electrophoresed the mixture in a native acrylamide gel; no effect of IHF on the mobility of the fragments was seen. To rule out the possibility that IHF bound to these fragments, but did not shift their mobility, we probed a transfer of the gel with antibody directed against IHF. No IHF was seen in association with the fragments, although control experiments using the H1 site of *attP* or a weaker IHF site found at the

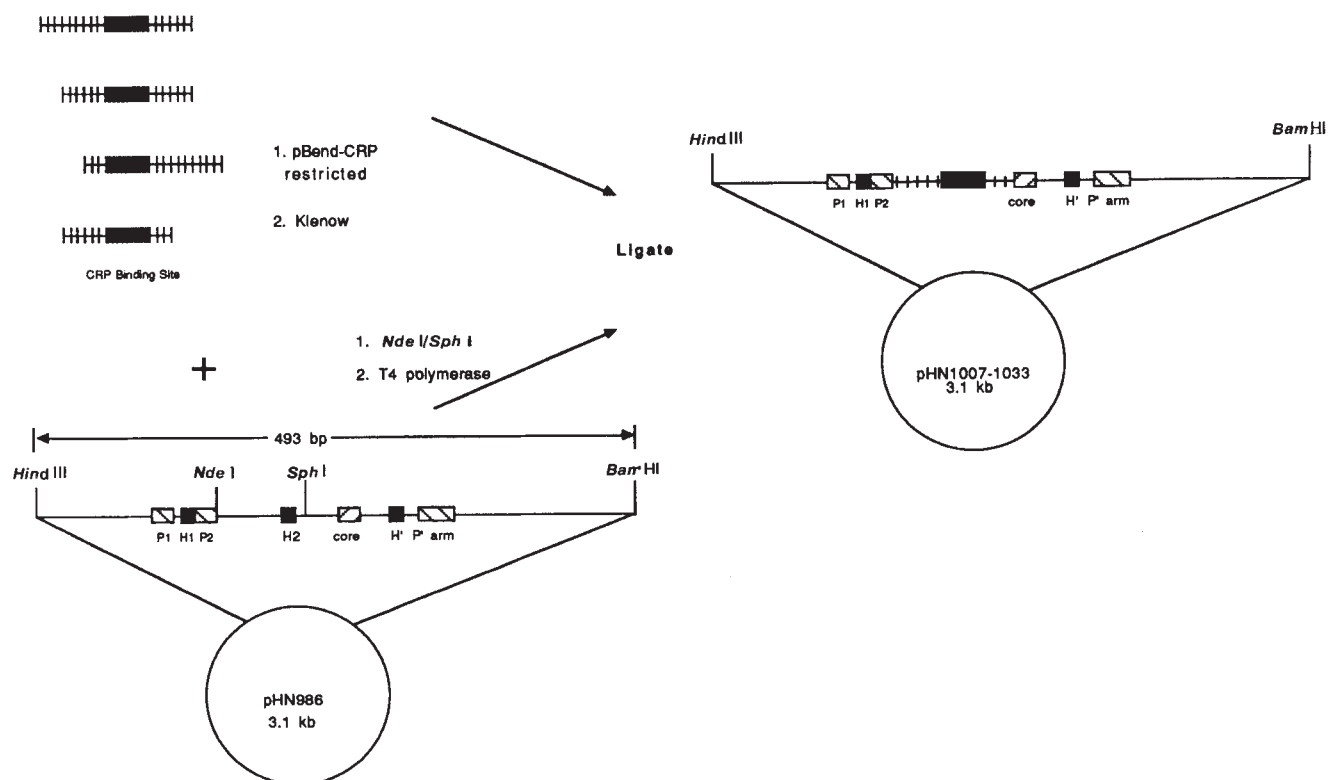


FIG. 1 Construction of chimaeras. Fragments containing a CRP site flanked by variable lengths of spacer DNA were generated by restriction of pBend-CRP, a plasmid in which the CRP site of the *lac* operon (black rectangle) is flanked by tandem repeats of a polylinker (individual sites represented by vertical hatch marks). These fragments, which have a size range from 64 to 106 bp, were used as substitutes for the 75 bp *Sph*I (–23) to *Nde*I (–98) segment of *attP*. Individual *attP* chimaeras (pHN1007–pHN1015) were isolated and sequenced. In addition to finding simple CRP inserts, some chimaeras contain an expected insert plus short stretches of DNA from the tandem repeats (a consequence of not purifying the population of inserts before ligation). From sequenced chimaeras additional clones (pHN1026–pHN1033) were generated by filling in a unique restriction site in the vicinity of the CRP binding site. In each case 4 bp were added to one side or the other of the CRP-site, increasing the distance of this site from either the core or the left side of *attP*.

METHODS. pBend1 and pBend-CRP were the gift of S. Adhya and J. Kim. pBend1 contains tandem direct repeats of 8 contiguous restriction sites

(5'-ACGCGTAGATCTGCTAGCATCGATCCATGGACTAGTCTCGAGGGATCC-3') separated by a single *Xba*I and *Sal*I site (see ref. 7 for a related construction). This cassette was cloned between the *Eco*RI-*Hind*III sites of a derivative of pBR322 that had been deleted for the 1.9 kb *Eco*RV to *Pvu*II segment. pBend-CRP was constructed by ligating a 38 bp fragment containing the *lac*CRP site (5'-AATTGCAATTAATGTGAGTTAGCTCACTCATTAGACCC-3'; the underlined portion is the -77 to -42 region of the *lac* operon; the sequence in bold lettering is the CRP-binding site) into the blunt ended *Xba*I site of pBend1. pHN986 was made by ligating the large *Nde*I-*Dra*I fragment of pUC-4K (ref. 21) to the *Hind*III-*Bam*HI fragment from phage λ that contains *attP* (coordinates 27,479–27,972). The *attP* segment came from pCB21 (ref. 22) and has an artificial *Sph*I site which is phenotypically silent for recombination. pHN1006 is identical to pHN986 except that it does not contain the *Sph*I site and there is a 4 bp change in the H2 IHF site which inactivates IHF binding⁹. The complete sequence of the *attP* region of the chimaeric plasmids is available upon request.

boundary of IS1 (ref. 17) gave readily detectable signals (data not shown). In a second series of experiments we determined that the recombination of our best chimaeras requires no more IHF than does recombination of wild-type *attP* (data not shown). That the chimaeras continue to require IHF reflects their dependence on the remaining sites, H1 and H'. The lack of an enhanced requirement for IHF, however, argues against the possibility that our chimaeras are active because they contain or create weak IHF binding sites.

Taken together, these experiments confirm our conclusion that the chimaeras create functional *attP* simply because the bend induced by IHF at H2 has been replaced by another source of bending. We believe that the strategy of 'bend swap' experiments is particularly powerful in analysing effects in multi-protein arrays. On the basis of experiments that either altered the protein, the binding site, or the spacing of the array around the binding site, others have suggested^{5,18,19} that bending by sequence-specific DNA binding proteins is a critical element for function. In each of these cases, however, it is hard to rule out the possibility that altered function follows from an altered protein-DNA or protein-protein contact rather than from a primary change in the conformation of DNA. By contrast, the

TABLE 2 Integrative recombination *in vivo* and *in vitro*

<i>attP</i>	Phenotype	Recombination (% of wild type)			
		<i>in vivo</i>		<i>in vitro</i>	
		(a)	(b)	(c)	(d)
pHN986*	wt	(100)	(100)	(100)	(100)
pHN1006	H2 ⁻	3	4	10	16
pHN1053	H2 ⁻	2	5	0.3	<0.1
pHN1043	<i>attP</i> -K	2	—	—	<0.1
pHN1045	<i>attP</i> -K	175	90	65	79
pHN1050	<i>attP</i> -K	12	—	—	16

In vivo recombination between the *attP* plasmids listed and a derivative of phage lambda that carries *attB* was performed as described⁹. The extent of recombination was measured as the per cent of phage progeny that acquired kanamycin resistance. Appropriate controls showed that this capacity depends on the presence of an attachment site in the plasmid and an *int* gene on the phage (not shown). *In vitro* recombination was performed and quantitated as described in Fig. 2.

*, % recombination of pHN986 for the separate experiments shown in columns (a), (b), (c), and (d) was 2.8, 10.9, 57 and 19 respectively.

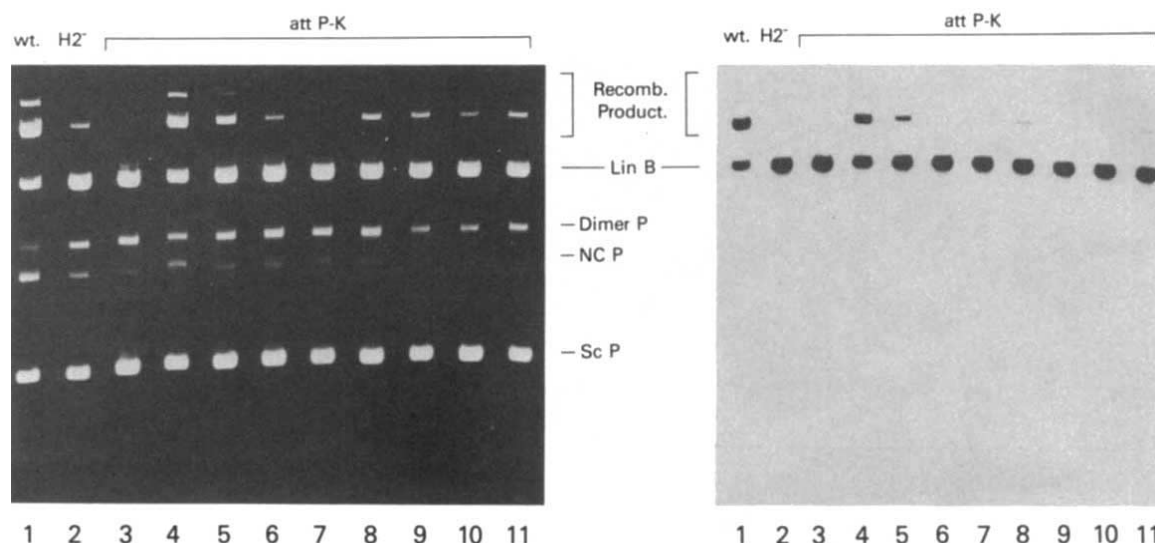


FIG. 2 *In vitro* recombination of *attP-K* chimaeras. The left panel shows the ethidium bromide stained agarose and the right panel shows the autoradiogram of the same gel. *attP*s used in each reaction are as follows: pHN986 (lane 1), pHN1006 (lane 2), pHN1044 (lane 3), pHN1045 (lane 4), pHN1046 (lane 5), pHN1047 (lane 6), pHN1048 (lane 7), pHN1049 (lane 8), pHN1050 (lane 9), pHN1051 (lane 10), pHN1052 (lane 11). *attP* phenotypes are given above each lane and the position of the substrates and products is indicated in the centre.

METHODS. *In vitro* recombination²² used 12.5 $\mu\text{g ml}^{-1}$ of each *attP* plasmid primarily as the supercoiled monomer (Sc P) with some nicked circular form (NC P) and dimer (Dimer P) present. All reactions contained an equal amount of the linear form *attB* (Lin B, pBB105 linearized with *EcoRI*, and end labelled with ³²P), purified IHF (20–30 nM) and purified Int (70–140 nM) in 50 mM Tris-HCl (pH 7.8), 70 mM KCl, 5 mM spermidine, 0.1 mM EDTA, 50 $\mu\text{g ml}^{-1}$ BSA, 1% glycerol in a final volume of 20 μl . Reactions were carried out at 25 °C for 45 min and analysed on 0.7% agarose gels. Recombination was quantitated using an automated beta detector (AMBIS) to determine the percentage of *attB* substrate converted into product. Construction of *attP-K* clones followed the strategy diagrammed for *attP-CRP*. A 77 bp blunt ended

fragment containing 6'A tracts' (5'-AATTC-(CGGGCAAAAAACGGCAAAAA)₃-CGGGGAATT-3'; ref. 16) was inserted at the *XbaI* site of pBend1 to create pHN1036. Fragments were liberated from pHN1036, cloned into pHN986 and sequenced as above (pHN1041–pHN1046). Additional +4 bp variants of the chimaeras were also generated (pHN1047–pHN1051) as described in Fig. 1. The insert sizes (bp) for plasmids pHN1041–pHN1051, respectively, are 132, 126, 114, 109, 144, 127, 136, 136, 113, 148, and 131. The distance (bp) between the presumptive bend centre (the centre of the 'A tracts') and the attachment site arm (see Table 1) for pHN1041–pHN1051 are 71, 55, 54, 48, 88, 79, 71, 75, 48, 92, and 83. In plasmids pHN1041, pHN1044, pHN1045, pHN1047, pHN1048, pHN1049 and pHN1050 the 'A' rich strand of the insert is continuous with the top strand of *attP* whereas in pHN1042, pHN1043, pHN1046, and pHN1051 the inserts are in the opposite orientation. pHN1052 was created by removing the *XbaI* insert that contains the 'A tracts' from pHN1045 and religating it in the opposite orientation. pHN1053 was constructed by inserting a blunt ended *TaqI* fragment from phage lambda (coordinates 44,667–44,811) between the *NdeI* and *SphI* sites of pHN986.

replacement strategy, especially the replacement of a protein-induced bend by a sequence-directed bend, seems less beset by alternative interpretations. It remains to be seen whether such replacements would be successful at the remaining IHF sites within *attP*. Even if IHF is needed to form protein-protein interactions at another site, we believe that our data establish the principle that, in building a functional nucleoprotein array, phage λ has evolved to use an accessory protein to change the conformation of DNA. Although we do not know how the bend is actually used, we are attracted to the idea that the bend acts as an architectural element. That is to say, by bringing distant parts of *attP* closer together, this bending could facilitate the interaction of other components of the intasome^{10,13,20}. Indeed, Landy and coworkers¹⁹ have recently shown that IHF assists the communication between Int bound to different segments of an attachment site. Although such experiments leave open the possibility that IHF acts by contacting Int bound to the arm so as to improve its affinity for the core region, it has been suggested from these data that the role of IHF is to bend the DNA¹⁹, a proposal entirely consistent with the conclusions of the present work.

In its size and complexity of binding sites, *attP* resembles other important loci of DNA metabolism in both prokaryotes and eukaryotes. At loci such as origins of replication, transcriptional control region and others, the same strategem used by the phage integration system may also apply: accessory proteins

that bend DNA may help in the construction of condensed nucleoprotein arrays. In testing for such a possibility, the bend-replacement approach that we have introduced in this paper should be very useful. □

Received 30 June; accepted 1 August 1989.

1. Wu, H.-M. & Crothers, D. M. *Nature* **308**, 509–513 (1984).
2. Zahn, K. & Blattner, F. R. *EMBO J.* **4**, 3605–3616 (1985).
3. Mukherjee, S., Patel, I. & Bastia, D. *Cell* **43**, 189–197 (1985).
4. Shuey, D. J. & Parker, C. S. *Nature* **323**, 459–461 (1986).
5. Hatfull, G. F., Noble, S. M. & Grindley, N. D. F. *Cell* **49**, 103–110 (1987).
6. Vignais, M.-L. & Sentenac, A. *J. biol. Chem.* **264**, 8463–8466 (1989).
7. Zwieb, C., Kim, J., & Adhya, S. *Genes Dev.* **3**, 606–611 (1989).
8. Craig, N. L. A. *Rev. Genet.* **22**, 77–105 (1988).
9. Gardner, J. F. & Nash, H. A. *J. molec. Biol.* **191**, 181–189 (1986).
10. Robertson, C. A. & Nash, H. A. *J. biol. Chem.* **263**, 3554–3557 (1988).
11. Thompson, J. F. & Landy, A. *Nucleic Acids Res.* **16**, 9687–9705 (1988).
12. Echols, H. *Science* **233**, 1050–1056 (1986).
13. Richet, E., Abcarian, P. & Nash, H. A. *Cell* **46**, 1011–1021 (1986).
14. Kosturko, L. D., Daub, E. & Murialdo, H. *Nucleic Acids Res.* **17**, 317–334 (1989).
15. Liu-Johnson, H.-N., Gartenberg, M. R. & Crothers, D. M. *Cell* **47**, 995–1005 (1986).
16. Koo, H.-S. & Crothers, D. M. *Proc. natn. Acad. Sci. U.S.A.* **85**, 1763–1767 (1988).
17. Gamas, P., Chandler, M. G., Prentki, P. & Galas, D. J. *J. molec. Biol.* **195**, 261–272 (1987).
18. Hübner, P., Haffter, P., Iida, S. & Arber, W. *J. molec. Biol.* **205**, 493–500 (1989).
19. de Vargas, L. M., Kim, S. & Landy, A. *Science* **244**, 1457–1461 (1989).
20. Stenzel, T. T., Patel, P. & Bastia, D. *Cell* **49**, 709–717 (1987).
21. Taylor, L. A. & Rose, R. E. *Nucleic Acids Res.* **16**, 358 (1988).
22. Kitts, P. A. & Nash, H. A. *J. molec. Biol.* **204**, 95–107 (1988).

ACKNOWLEDGEMENTS. We thank Drs R. Weisberg, J. Gardner and S. Adhya for discussions, J. Harman for a gift of CRP protein, J. Trempey and J. Winkelman for help with the western blots, C. Robertson for technical assistance and M. Gellert and M. Ptashne for critical review of the manuscript.

Phasing of protein-induced DNA bends in a recombination complex

Ursula K. Snyder*, John F. Thompson*†
& Arthur Landy*

* Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912, USA

† Department of Molecular Genetics, Pfizer Central Research, Groton, Connecticut 06340, USA

MANY of the structures responsible for replication, transcription initiation and recombination arise from complex sets of protein-protein interactions and the folding of DNA in three dimensions, with protein-induced bending of DNA often playing an integral role. The magnitude and orientation of DNA bending induced by various single proteins has been estimated by gel mobility shift methods¹⁻⁴ and by modelling of crystallographic data⁵. The site-specific recombination by which bacteriophage lambda (phage λ) integrates into the chromosome of its host *Escherichia coli* requires a host protein, 'integration host factor' (IHF), which is known to be able to bend the DNA to which it binds. To determine the three-dimensional path of DNA within the higher order structure responsible for phage λ site-specific recombination^{6,7}, we have determined the relative direction of IHF-induced bending at each of the three binding sites within the complex^{8,9}. IHF, which appears to bend DNA by more than 140°, is a major determinant of the DNA path in the recombination complex³ and is also involved in a wide range of other cellular events¹⁰.

Two different methods were used to determine the phase of a pair of IHF-induced bends. One method depends upon alterations in electrophoretic mobility as a function of spacing between the IHF binding sites^{1,2}; the other depends on alter-

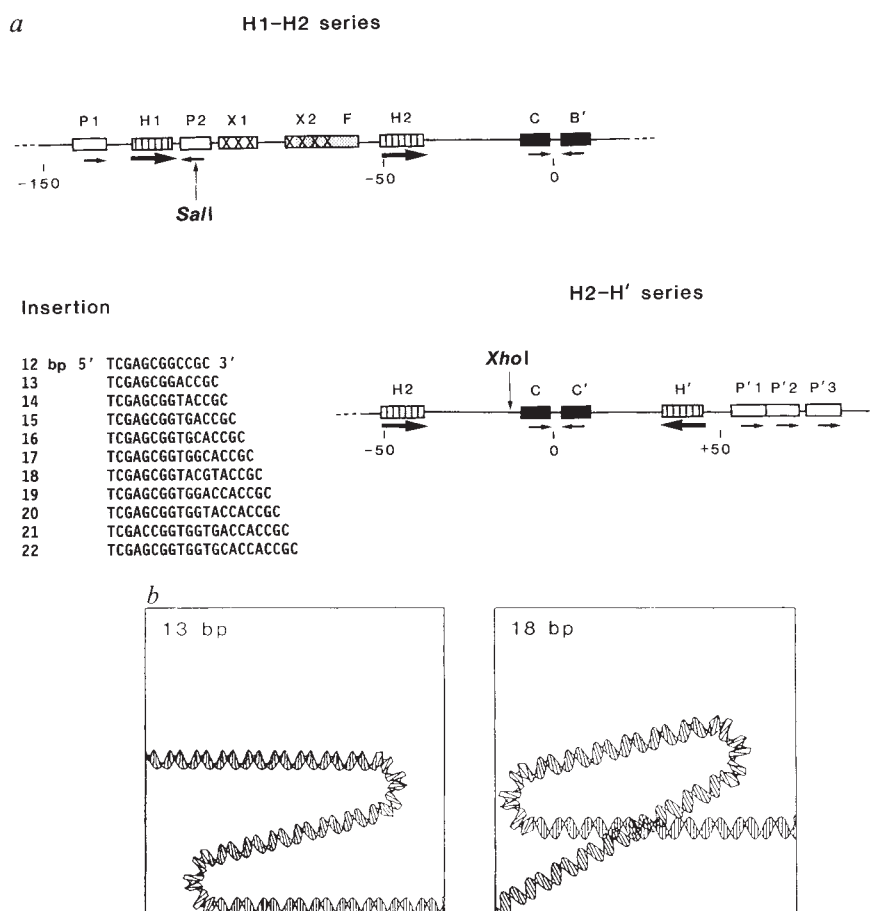
ations in the stability of IHF-DNA complexes as a function of spacing. Phasing information derived from the cyclical variation of complex stability is independent of electrophoretic mobility and circumvents complications caused by intrinsic curvature. Results from these two methods are in excellent agreement and lead to a model for the recombination complex formed with the phage λ integration site *attP*.

Two sets of 12 DNA fragments derived from *attP* were constructed with each successive fragment containing one additional base pair (bp) between the pairs of IHF binding sites H1 and H2, or H2 and H' (Fig. 1a). Insertion of 1 bp rotates the two DNA-binding sites with respect to each other by an average of 34° (ref. 11) and also changes the position of the two fragment termini with respect to one another in three-dimensional space. Incremental insertions of single base pairs through a full helical turn between two DNA bends produces a cyclic variation in the electrophoretic mobility of fragments in polyacrylamide gels. The minimum and maximum mobilities occur for those fragments that have two bends in phase, or out of phase, respectively^{1,2}. With the two bends in phase, a closed compact structure is predicted; when the two bends are out of phase the structure is open and extended (Fig. 1b). These models suggested that there might be a difference in the stability of the IHF-DNA complex as a function of the relative orientation of the DNA arms (caused by steric hindrance) as well as variation in electrophoretic mobility.

When IHF is bound to any of the fragments, bands with three different mobilities are observed (Fig. 2a, 2b). The fastest migrating band corresponds to unbound DNA. As observed previously^{12,13}, *attP* has intrinsic curvature and the unbound fragments have an altered mobility as a result of changing the phasing between multiple A-tracts. Because this altered mobility is not in phase with the protein-induced bends, it is difficult to rigorously separate these two effects. The two slower migrating

FIG. 1 a, The protein-binding region of *att* fragments containing two IHF binding sites. The parent fragment in each series is derived from *attP* and contains the protein binding sites for Int (P1, P2, C, B', C, C', P'1, P'2 and P'3), IHF (H1, H2, and H'), Xis (X1, X2) and Fis (F) (see also Fig. 4). Taken together, the parent fragments span the full *attP*. b, the path of DNA predicted for fragments containing a 13 bp (left panel) and an 18 bp (right panel) insertion between two occupied IHF binding sites. The figures were generated with the DNA bending program from DNASTAR Inc. (Madison) based on the length of restriction fragments used in this study (419–516 bp), the distance between the IHF binding sites (91 and 96 bp for the 13 bp and 18 bp insertion respectively), and the magnitude of the IHF-induced bends (> 140°) (ref 3).

METHODS. H1-H2 series: oligomers of 12–22 bp were inserted into the *Sal*I site of a pJT33 (ref. 19) derivative in which a 994 bp *Bam*HI-StyI fragment in the pBR327 backbone was deleted. The parent and the set of 11 plasmids were cut with *Eco*RI and *Pst*MI to generate fragments of 419, 431–441 bp. H2-H' series: the same oligomers were inserted into an *Xho*I site of a pJT96 (ref. 13) derivative in which the 3,533 bp *Asp*718-*Pst*I fragment was ligated to the 1,129 bp *Pst*I-*Bam*HI fragment of pBR327. The parent and the set of 11 plasmids were cut with *Eco*RV and *Bam*HI to generate fragments of 494, 506–516 bp. All plasmids were sequenced with a kit from U.S. Biochemical Corp. (Cleveland, Ohio).



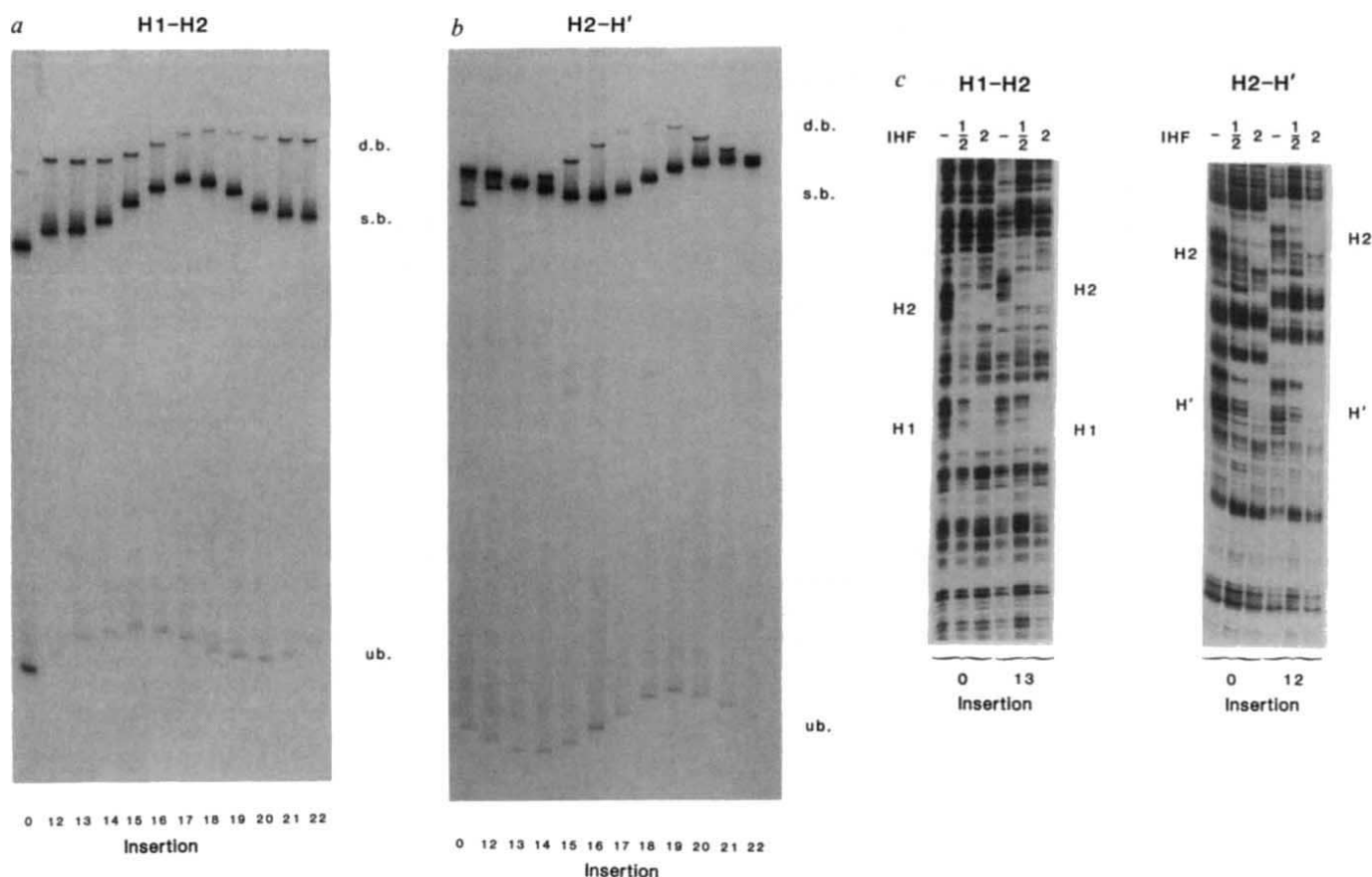


FIG. 2 Polyacrylamide gel electrophoresis of IHF-DNA complexes. *a*, H1-H2 series. *b*, H2-H' series. The three bands correspond to unbound DNA (ub.), DNA with a single IHF bound (s.b.), and DNA with IHF bound to both sites (d.b.). *c*, Nuclease protection assay of IHF-DNA complex. The degree of protection of IHF binding sites was used to determine site occupancy. The DNA isolated from the single-bound species shows partial protection of both binding sites and the DNA from the double-bound species shows complete protection. The partial protection exhibited by the single-bound species is caused by IHF binding to each site for a time proportional to the binding site affinity³⁰. The observed relative affinities of the IHF sites,

$H2 \geq H' > H1$, are in agreement with earlier studies⁸.

METHODS. Fragments were purified after end-labelling at the *EcoRI* (H1-H2 series) or the *BamHI* (H2-H' series) sites using the Klenow fragment of DNA polymerase and [α -³²P]dATP. Binding and electrophoresis of IHF-DNA complexes were as described³. The results using 1/2 unit of IHF are shown. For the nuclease protection experiment, IHF-DNA complexes were digested with neocarzinostatin and electrophoresed on 5% polyacrylamide gels. DNA associated with the single- and double-bound species (determined by IHF titration) was electroeluted and electrophoresed on 7.5% polyacrylamide, 8 M urea gels.

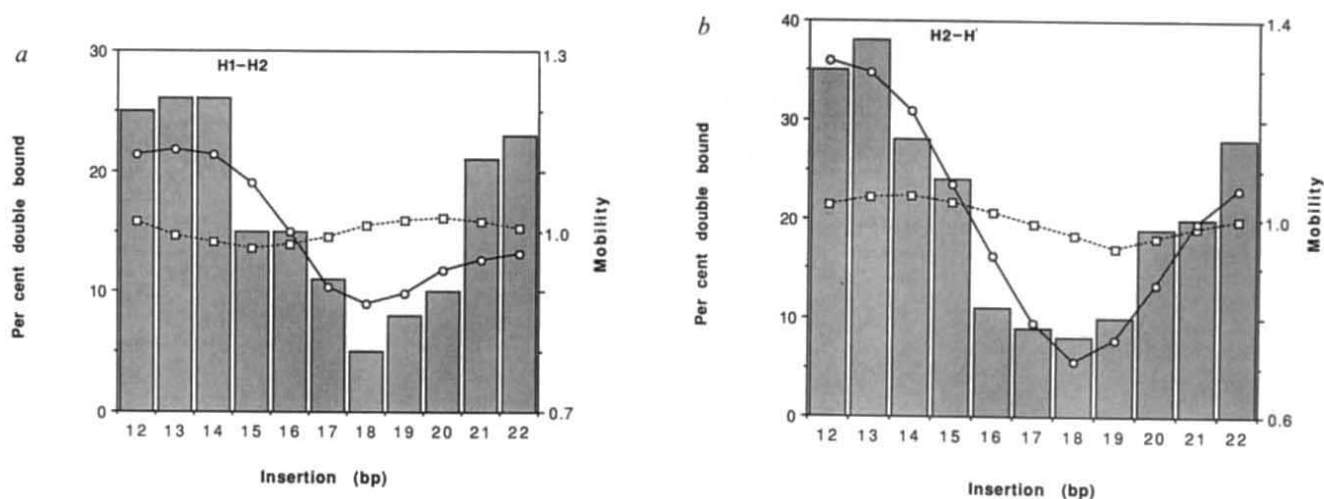


FIG. 3 Stability and relative mobility of the double bound species plotted as a function of insertion length. *a*, H1-H2 series. *b*, H2-H' series. The bar graphs depict the percent of radioactivity transferred from the labelled substrate to the species having IHF bound to both sites, as determined by scanning with a laser densitometer. The proportion of radioactivity associ-

ated with the double-bound species varied with the same helical periodicity for all the concentrations of IHF tested (1/4-2 units). The relative mobility is calculated as the fractional variation in electrophoretic mobility normalized to the average for the set of 11 fragments. $\circ$, relative mobility of the doubly bound species; $\square$, relative mobility of the unbound DNA.

bands correspond to DNA with IHF bound to either one or to both sites on the molecule.

To identify the single-bound and the double-bound species, IHF titrations and nuclease protection experiments were performed. The IHF titration indicated that the slowest migrating band for each fragment of the H1-H2 series contained IHF bound to both sites. This was also the case for the H2-H' series, with the exceptions of the fragments with insertions of 0, 12, or 13 bp, for which the faster migrating band contained IHF bound to both sites. These results were confirmed by nuclease protection experiments with the single- and double-bound complexes (Fig. 2c).

The proportion of DNA with IHF bound to both sites varies as a function of the spacing between the two sites (Fig. 3a, b). On the same graph is a plot of the relative electrophoretic mobility of the double-bound species as a function of spacing. Both parameters exhibit the same helical periodicity, as predicted for a dependence on the relative orientation of the IHF-induced bends.

For the fragments with IHF bound to both sites, the greatest fraction of label is associated with those complexes having the highest relative mobility and hence the most extended structure. As the relative orientation of the bends is changed to produce a more compact structure with the ends of the DNA in the same vicinity, there is a decrease in the stability of the complex as a result of the interfering DNA. To verify this interpretation of the results, we shortened the length of DNA flanking the H1 and H2 binding sites for the fragments with the 13 bp and 18 bp insertions. Removing 97 bp and 54 bp from the left and right ends, respectively, eliminated the difference in binding affinities (data not shown).

The close correspondence between the complex-stability and mobility results corroborates the interpretations of these types of analyses and is consistent with the earlier measurements of IHF-induced bends in phage λ *attP* (ref. 3). Because the lowered stability of the double-bound complexes is probably due to close contacts that are made by the distal arms of the DNA fragment, the effect of phasing may only be observed with appropriately spaced binding sites and proteins that bend the DNA by more than 90°, as does IHF. These stretches of DNA must bend or twist to avoid each other, thus distorting either the DNA or the optimal protein-induced bend, thereby decreasing the stability of the complex. Induced bends of greater than 90° may be common, as IHF, FIS, CAP, Xis and γ d resolvase all fall into this category^{3,5,14,15}.

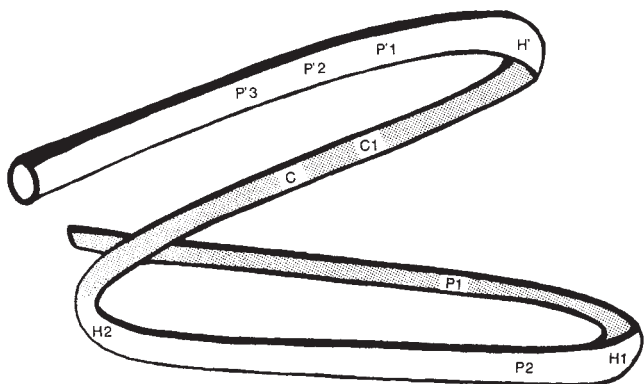


FIG. 4 Model of the three-dimensional path of *attP* DNA. The drawing shows the path of *attP* DNA that would result from IHF binding to the three sites given a relative phase of 103° between adjacent bends and a magnitude of 140° for each bend. There are two types of Int binding sites: arm-type (P1, P2, P1', P2', P3') that are recognized by the N-terminal domain of Int, and core-type sites (C and C') that are recognized by the C-terminal domain, and are the sites of strand exchange during recombination²⁹. Int does not induce significant bends in the DNA³, but has the capacity to bridge two DNA binding sites through its autonomous binding domains²⁸.

The complex stability and mobility data indicate that the IHF binding sites in *attP* are not in phase. For both the H1-H2 and the H2-H' series, the addition of 18 bp places the two binding sites on the same face of the DNA helix. If one takes the value of 10.5 bp per turn for the average helical screw¹⁶⁻¹⁸, 3 more bp (corresponding to 103°) are required to bring the binding sites back to their natural orientation. Thus, the bend at H2 is ~100° out of phase relative to H1. Although the orientation of H2 and H' are opposite, these two bends are also ~100° out of phase (in the same rotational sense). Because the fragments were derived from *attP*, the effect of the intrinsic curvature of *attP* on the IHF-induced bends is included. Intrinsic curvature may play a role in aligning certain sites more specifically, and may also decrease the binding energy necessary for IHF binding at the H2 and H' sites, relative to H1, which is in a region where intrinsic curvature is absent^{12,13}.

There is considerable evidence^{3,19-25} that the integration of phage λ into the *E. coli* chromosome requires the formation of a higher-order complex of *attP* and the proteins IHF and Integrase (Int), which carries out the cutting and rejoining of *att* site DNA during recombination^{26,27}. We have constructed a model of the three-dimensional structure of *attP* using the relationship between the IHF-induced bends described above (Fig. 4). Addition of the three phased bends results in a left-handed coil structure as predicted on the basis of topological analyses^{24,25}. (This is topologically equivalent to a right-hand plectonemic wrap.) The degree to which the bends are out of phase implies an extended structure in which the P and P' arm-type Int binding sites are far apart in three dimensions. This structure does not take into account any conformational changes induced by supercoiling or Int, both of which are likely to condense the extended structure so that distal regions of the complex could communicate.

Although we are not yet able to incorporate all perturbations of the DNA path, these experiments lead to a preliminary model of the higher order structure involved in phage λ integrative recombination that correctly predicts topological features^{24,25}, and also provides a DNA framework for the known bridging capacity of Int^{28,29} and long range interactions within the intasome^{19,20,22}. □

Received 12 May; accepted 7 August 1989.

1. Zinkel, S. S. & Crothers, D. M. *Nature* **328**, 178-181 (1987).
2. Salvo, J. J. & Grindley, N. D. F. *Nucleic Acids Res.* **15**, 9771-9779 (1987).
3. Thompson, J. F. & Landy, A. *Nucleic Acids Res.* **16**, 9687-9705 (1988).
4. Johnson, R. C., Glasgow, A. C. & Simon, M. I. *Nature* **329**, 462-465 (1987).
5. Weber, I. T. & Steitz, T. A. *Proc. natn. Acad. Sci. U.S.A.* **81**, 3973-3977 (1984).
6. Landy, A. A. *Rev. Biochem.* **58**, 913-949 (1989).
7. Thompson, J. F. & Landy, A. in *Mobile DNA* (eds Berg, D. E. & Howe, M. M.) 1-22 American Society for Microbiology, Washington D.C., 1989).
8. Craig, N. L. & Nash, H. A. *Cell* **39**, 707-716 (1984).
9. Robertson, C. A. & Nash, H. A. *J. biol. Chem.* **263**, 3554-3557 (1988).
10. Friedman, D. I. *Cell* **55**, 545-554 (1988).
11. Kabsch, W., Sander, C. & Trifonov, E. N. *Nucleic Acids Res.* **10**, 1097-1104 (1982).
12. Ross, W. & Landy, A. *J. molec. Biol.* **156**, 505-529 (1982).
13. Thompson, J. F., Mark, H. F., Franz, B. & Landy, A. in *DNA Bending and Curvature* (eds Olson, W. K., Sarma, M. H., Sarma, R. H. & Sundaralingam, M.) 119-128 (Adenine Press, Guildford, NY, 1988).
14. Liu-Johnson, H.-N., Gartenberg, M. R. & Crothers, D. M. *Cell* **47**, 995-1005 (1986).
15. Salvo, J. J. & Grindley, N. D. F. *EMBO J.* **7**, 3609-3616 (1988).
16. Wang, J. *Proc. natn. Acad. Sci. U.S.A.* **76**, 200-203 (1979).
17. Trifonov, E. N. & Bettecken, T. *Biochemistry* **18**, 454-456 (1979).
18. Rhodes, D. & Klug, A. *Nature* **286**, 573-578 (1980).
19. Thompson, J. F., Moitoso de Vargas, L., Skinner, S. E. & Landy, A. *J. molec. Biol.* **195**, 481-493 (1987).
20. Thompson, J. F., Snyder, U. K. & Landy, A. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6323-6327 (1988).
21. Hsu, P.-L., Ross, W. & Landy, A. *Nature* **285**, 85-91 (1980).
22. Richet, E., Abcarian, P. & Nash, H. A. *Cell* **46**, 1011-1021 (1986).
23. Better, M., Lu, C., Williams, R. C. & Echols, H. *Proc. natn. Acad. Sci. U.S.A.* **79**, 5837-5841 (1982).
24. Spengler, S. J., Stasiak, A. & Cozzarelli, N. R. *Cell* **42**, 325-334 (1985).
25. Griffith, J. D. & Nash, H. A. *Proc. natn. Acad. Sci. U.S.A.* **82**, 3124-3128 (1985).
26. Craig, N. L. & Nash, H. A. *Cell* **35**, 795-803 (1983).
27. Hsu, P.-L. & Landy, A. *Nature* **311**, 721-726 (1984).
28. Moitoso de Vargas, L., Kim, S. & Landy, A. *Science* **244**, 1457-1461 (1989).
29. Moitoso de Vargas, L., Pargellis, C. A., Hasan, H. M., Bushman, E. W. & Landy, A. *Cell* **54**, 923-929 (1988).
30. Prentki, P., Chandler, M. & Galas, D. J. *EMBO J.* **6**, 2479-2487 (1987).

ACKNOWLEDGEMENTS. We thank Lucy Rodrigues and Bonnie Tracy for technical assistance, Joan Boyles for preparation of the manuscript and William Montigny and Simone Nunes-Duby for suggestions. This work was supported by grants from the NIH (A.L.) and the ACS (J.T.).

Please follow these guidelines so that your manuscript may be handled expeditiously.

Nature is an international journal covering all the sciences. Contributors should therefore bear in mind those readers for whom English is a second language and those who work in other fields. Please write clearly and simply, avoiding unnecessary technical terminology. *Nature's* staff will edit manuscripts to those ends if necessary. Contributors should check their proofs carefully.

Because of the competition for space, many of the papers submitted for publication cannot be accepted. For this reason, and because brevity is a great assistance to readers, papers should be as brief as is consistent with intelligibility. Please note that one printed page of *Nature*, without diagrams or other interruptions of the text, has fewer than 1,300 words.

CATEGORIES OF PAPER

Review Articles survey recent developments in a field. Most are commissioned, but suggestions are welcome in the form of a one-page synopsis addressed to the Reviews Coordinator. Length is negotiable in advance but must not exceed six pages of *Nature*.

Articles are research reports whose conclusions are of general interest and which are sufficiently rounded to be a substantial advance in understanding. They should not have more than 3,000 words of text or more than six display items (figures and tables) and should not occupy more than five pages of *Nature*.

Articles should be accompanied by a heading of 50–80 words written to advertise their contents in general terms, to which editors will pay particular attention. A heading (printed in italic type) is not an abstract and should not usually contain numbers or measurements. The study should be introduced in more detail in the first two or three paragraphs, which should also briefly summarize its results and implications.

Articles may contain a few subheadings of two or three words. The meaning of the text should not depend on the subheadings, whose function is to break up the text and to point to what follows. There should be fewer than 50 references.

Letters to Nature are short reports of outstanding novel findings whose implications are general and important enough to be of interest to those outside the field. Letters should not have more than 1,000 words of text or more than four display items and should not occupy more than two pages of *Nature*. The first paragraph should describe, in not more than 150 words, the origins and chief conclusions of the study. Letters should not have subheadings or more than 30 references.

Commentary articles deal with issues in, or arising from, research that are also of interest to readers outside research. Some are commissioned, most are unsolicited. They are normally between one and four pages of *Nature* in length.

News and Views articles are intended to inform non-specialist readers about a recently published advance. Suggestions should be made to the News and Views Editor. Illustrations are welcome. Proposals for meeting reports should be agreed in advance.

Scientific Correspondence is for the discussion of scientific matters, including contributions published in *Nature*. Priority is given to contributions of less than 500 words and five references. Figures are welcome.

Submission. All manuscripts may be submitted to either London or Washington. They should not be addressed to editors by name. Manuscripts or proofs sent by air courier to London should be declared as 'manuscripts' and 'value \$5' to prevent the imposition of import duty and value-added tax (VAT).

GENERAL

All contributions submitted for publication in *Nature* should conform with these rules.

Manuscripts should be typed, double-spaced, with a good-quality printer, on one side of the paper only. Four copies are required, each accompanied by lettered artwork. Four copies of half-tones should be included. Reference lists, figure legends and so on should be on separate sheets, all of which should be double-spaced and numbered. Copies of relevant manuscripts in press or submitted for publication elsewhere should be included, clearly marked as such. Revised and resubmitted manuscripts should also be clearly marked as such and labelled with their reference numbers.

Titles should say what the paper is about with the minimum of technical terminology and in fewer than 80 characters. Authors should avoid active verbs, numerical values, abbreviations and punctuation and should include one or two key words for indexing purposes.

Artwork should be marked individually and clearly with the author's name, figure number and, when known, manuscript reference number. Original artwork should be unlettered. Ideally, no figure should be larger than 28 by 22 cm. Figures with several parts are permitted only if the parts are closely related, either experimentally or logically. Suggestions for cover illustrations, with captions, are welcome. Original artwork (and one copy of the manuscript) will be returned when a manuscript cannot be published.

Colour Artwork. A charge of £500 a page is made for 4-colour figures. Inability to meet these costs will not prevent the publication of essential colour figures if the circumstances are explained.

Figure legends must not exceed 300 words and ideally should be much shorter, and should use telegraphic form wherever possible. The figure should be described first, then, briefly, the method. Reference to a method described elsewhere is preferable to a full description. Methods should not be described in detail in the text.

References should be numbered sequentially as they appear in the text, followed by those in tables and finally those in the figure legends. Reference numbers apply only to papers published or in the press, and a different number should be given to each paper cited. All other forms of reference (including unrefereed abstracts) should be included in the text as personal communication, manuscript in preparation or preprint (with number and institution where appropriate). Text should not be included in the reference list. References should be abbreviated according to the *World List of Scientific Periodicals* (fourth edition, Butterworth, London, 1963–65). The first and last page numbers should be cited. Reference to books should clearly indicate the publisher and the date and place of publication.

Abbreviations, symbols, units and Greek letters should be identified the first time they are used. Acronyms should be avoided as much as possible and, when used, defined. Editors will shorten words if necessary. In case of doubt, SI units should be used.

Footnotes should not be used except for changed addresses or to identify the corresponding author if different from the first-named.

Acknowledgements should be brief. Grant numbers and contribution numbers are not allowed.

Analytical ultracentrifugation reborn

H.K. Schachman

Analytical ultracentrifugation is still the best method for quantitative studies of the interactions between macromolecules. There will soon be a modern alternative to the ancient Beckman Model E.

FOR about a half century, beginning with the pioneering work of Svedberg and his collaborators in Uppsala, Sweden, the ultracentrifuge was arguably the most powerful tool available for the study of macromolecules of biological interest. By the 1930s, those workers (and others who spent a year or two at that Mecca) had already developed the principles and techniques of sedimentation velocity and sedimentation equilibrium¹.

The former led to the first reliable estimates of the shape of macromolecules by combining measurements of the sedimentation coefficient and the molecular weight with hydrodynamic theories for the frictional resistance of particles of different shapes as they migrated through aqueous solutions. Sedimentation equilibrium measurements based on rigorous thermodynamic principles yielded accurate values of the molecular weights of molecules, from sucrose to whole viruses with 'molecular weights' of as much as 5×10^7 .

The impetus to biological research resulting from the construction of a high-speed centrifuge capable of generating fields roughly 4×10^5 times that of gravity — coupled with ingenious optical systems that permitted molecules to be viewed directly as they moved through solvent — is virtually inestimable. It is hardly an exaggeration to state that the field of protein chemistry (in terms of molecular weights, subunit composition and association-dissociation equilibria) derives from the design and application of the ultracentrifuge². Perhaps the greatest surprise to the early pioneers was the demonstration that protein molecules were homogeneous and had well-defined molecular weights. This seminal conclusion from ultracentrifugal studies generated unbounded enthusiasm for structural investigations, leading ultimately to the elucidation of the sequence of amino acids in the polypeptide chains of innumerable proteins.

Because the oil turbine ultracentrifuge was extremely complicated and expensive, few laboratories in the world were equipped to construct and maintain them. In only a few years, however, the ingenuity of research workers resulted in the development of the air-driven ultracentrifuge³, leading rapidly to a doubling or more of the number of ultracentrifuges in routine operation. But this was still insufficient for the demands of research workers interested in the physical chemistry of proteins and nucleic acids. This

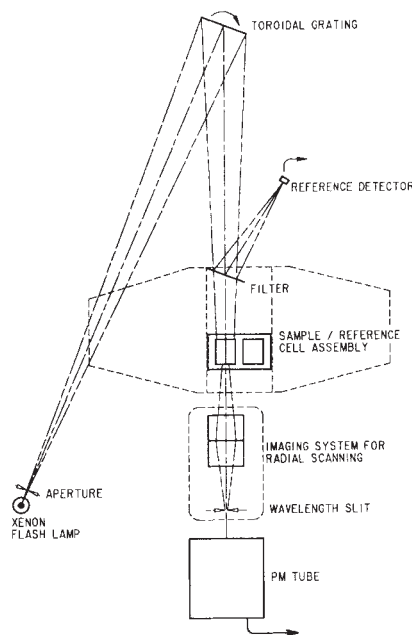


FIG. 1 The scanning absorption optical system of the Beckman Optima XL ultracentrifuge.

deficiency was remedied in 1948 by the development of an electrically driven commercial instrument⁴ with much greater reliability and ease of operation. Despite the cost of the instrument and the complexity of interpreting the wealth of information which could be deduced from sedimentation velocity and sedimentation equilibrium experiments, more than 2,000 commercial instruments were operating in laboratories throughout the world from 1950 to 1970.

But by 1980 — despite the almost frenetic activity devoted to the design of new cells, the incorporation of different optical systems, the development of additional theoretical treatments⁵⁻⁷, and the application of ultracentrifugation to a diverse host of important biological problems — the use of the instrument came to a rather abrupt end.

What happened? Were better, more reliable and more versatile techniques developed that could be used instead of ultracentrifugal methods? Or were the questions asked by protein chemists and molecular biologists in the 1980s so different that sedimentation techniques had become obsolete?

Not obsolete

To research workers interested in the absolute determination of the molecular weights and shapes of macromolecules, the association-dissociation equilibria of

oligomeric proteins, and the measurement of the interactions between dissimilar macromolecules, the ultracentrifuge is still the tool of choice. No other physical chemical tool surpasses the ultracentrifuge for these studies.

But molecular biologists in the early 1980s who were interested in molecular interactions between different proteins, or between proteins and nucleic acids, were content with 'yes or no' answers rather than equilibrium constants and free energy changes. For them, filter binding assays, Sephadex columns and polyacrylamide gels provided the desired information.

Now, however, with literally hundreds of proteins produced by the technique of site-directed mutagenesis awaiting characterization, precise techniques for physical chemical investigations are needed. Indeed, in a *News and Views* commentary two years ago, Gratzer⁸ discussed the use of analytical ultracentrifugation in studying the remarkable shape changes which some proteins exhibit in response to environmental perturbations: "The study of these phenomena has led to a return to the quantitative virtues of physical protein chemistry and a resurgence of ancient crafts: The whine of the Model E is once again to be heard in the land, like the voice of the turtle, to gladden the hearts of the old-timers." For the benefit of some readers, Gratzer added a footnote pointing out that the Model E is "not an early car, but the Beckman analytical ultracentrifuge".

Reinvention of a classic

Most 'old-timers' (though having fond memories of the Model E and in some cases still using it) are now excited about the recently announced plan by Beckman to make available a new analytical ultracentrifuge based on their Optima XL series.

Gone will be the noisy, old centrifuge drives with their relatively limited lifetimes and the requirement for a family of gears which permitted the rotor to spin at 70,000 r.p.m. even though the drive operated at a much lower speed. With their new, air-cooled induction motor, warranted for ten years, we look forward to smooth, constant-speed operation without the rotor precession that plagued workers in the past. Also, the ultracentrifuge will be much smaller than the Model E — a blessing to the research worker who almost invariably has too little available laboratory space. Gone also will be the

'medieval' temperature control system, with a needle at the base of the rotor dipping into a cup containing mercury to complete an electrical circuit. We all look forward to the disappearance of this archaic method of measuring and controlling the temperature of the rotor!

At the national meeting of the Biophysical Society this year, Beckman unveiled its new photoelectric scanning absorption optical system (Fig. 1), which will be completely contained within the vacuum chamber. It will have a range of wavelengths, sensitivity, linearity and accuracy that should permit routine studies at protein concentrations far lower than most workers would have dreamt possible. Moreover, the optical system should have sufficient resolution that, coupled to automatic data systems, the workers will obtain both integral curves (representing concentration versus distance) and derivative curves in the form of plots of gradient versus distance. Thus, those workers who are so accustomed to the 'peaks' produced directly by the schlieren optical system will have their equivalent with the added advantages of the discrimination and sensitivity of absorption optics. A mere change in the wavelength of the incident light over a broad range will provide information about the chemical composition of the sedimenting material. (In contrast, refractometric methods are incapable of providing such information.) Beckman plans also to incorporate a Rayleigh interference optical system so that the remarkable precision inherent in interferometry will be available to those workers interested in the highest accuracy of molecular weight determinations by the sedimentation equilibrium technique.

Coupled with all of these dramatic improvements will be new cells, rotors and — most important — automatic operation controlled by convenient, versatile computer programs which will also perform the many, diverse computations needed to determine molecular weights, sedimentation coefficients, homogeneity or heterogeneity of the macromolecules, and to analyse, in quantitative terms, association-dissociation equilibria. We can hardly wait. □

Howard K. Schachman is at the Department of Molecular Biology and the Virus Laboratory, University of California, Berkeley, California 94720, USA. For more information, fill in reader service number 100.

1. Svedberg, T. & Pedersen, K.O. *The Ultracentrifuge* (Clarendon, Oxford, 1940).
2. Schachman, H.K. *Ultracentrifugation in Biochemistry* (Academic, New York, 1959).
3. Beams, J.W. & Pickels, E.G. *Rev. Sci. Instrum.* **6**, 299 (1935).
4. Pickels, E.G. *Machine Design* **22**, 102 (1950).
5. Williams, J.W. (ed.) *Ultracentrifugal Analysis in Theory and Experiment* (Academic, New York, 1963).
6. Yphantis, D.A. (ed.) *Advances in Ultracentrifugal Analysis* (Ann. N.Y. Acad. Sci. **164**, 1969).
7. Fujita, H. *Foundations of Ultracentrifugal Analysis* (Wiley, New York, 1975).
8. Gratzel, W. *Nature* **328**, 669 (1987).

The best of British!

Visitors to British Lab Week, in London, can expect to see a wide range of laboratory products: from gel documentation systems to water purifiers that keep water particle-free.

ACCORDING to Ultra-Violet Products Ltd, the new video system for **electrophoresis gel documentation** can produce economical high-quality images in less than 10 seconds (*Reader Service No. 101*). Designed specifically for photography of low-light fluorescent patterns such as



Electrophoresis gel documentation from UV Products.

ethidium bromide-stained DNA gels or TLC plates, the system costing under £7,000 (UK) comprises CCD-camera, stand, UV transilluminator with filters, optics and control unit, nine-inch video monitor and video printer. The gel is placed on the transilluminator and the desired image is selected by moving the camera up and down on its stand. The integrated image is stored in a frame buffer and displayed on the video monitor, at which time the user can focus the image, use contrast amplification, edge enhancement or baseline subtraction. At the touch of a button, a 10 × 7.5-cm hard copy of the stored image can be printed on thermal paper, either as a positive or negative. Alternatively, the stored image can be transmitted to a PC for further processing. UVP will be on stand A248.

New from Jones Chromatography Ltd, is the aptly named Janitor — an ancillary module for HPLC machines designed to **flush out corrosive buffers and solvents** (*Reader Service No. 102*). The £550 (UK) Janitor module is connected to the HPLC pump inlet and allows the pump to deliver up to two solvents other than the mobile phase for preselected intervals. The

system operates by using timed switching valves and relays which are activated at the end of a series of chromatographic runs. After completion of the flush cycle, the Janitor relay powers down the system. According to the company, the Janitor will prolong the life of the HPLC column and detector lamp, and will reduce wear and corrosion on the HPLC system. Jones Chromatography can be seen on stand A400.

ABB Metrawatt Ltd's SE571 **storage oscilloscope** will be on show on stand G632 (*Reader Service No. 103*). The SE571 has eight built-in logic channels and can acquire both analogue and digital signals simultaneously. A video controller and rapid measured data conditioning facility allow a real-time screen display of waveform signals. The company says, data can be printed in less than 10 seconds as either a time diagram or data lists on the system's integrated printer. The instrument also features an auto-ranging facility for the control of amplification and time-base settings for both measuring channels. A computer can be connected to the SE571 via an IEEE 488 interface to control the measuring sequence. The SE571 can store

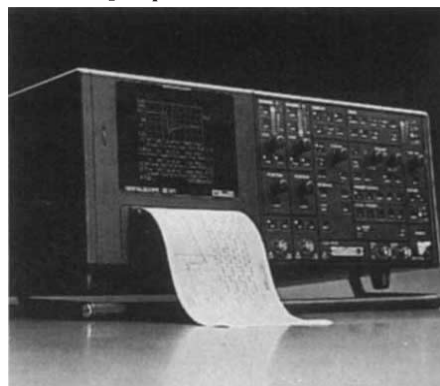


ABB Metrawatt's oscilloscope has 8 channels, up to ten sets of instrument settings, including measurement range, time-base settings, trigger levels and test signals.

A portable CO2001 **dryer that removes CO₂ and H₂O** from the compressed air for use in critical applications such as FT-IR and NMR spectrometers can be seen on Complete Protection Ltd's stand, number K1004 (*Reader Service No. 104*). The £895 (UK) CO2001 dryer can achieve CO₂ removal to less than 1 p.p.m. and a pressure dew point of less than -70 °C, says the company. The 843 × 305 × 106-mm unit offers particle removal to 0.2 microns and

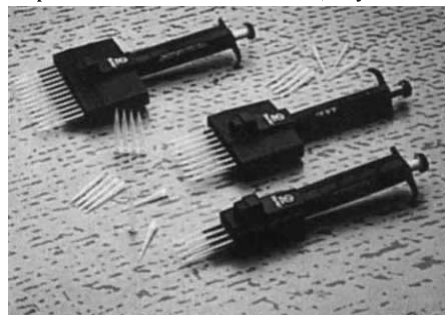


The CO2001 removes CO₂ and H₂O from compressed air.

a maximum output rate of 18 litres per minute, says Complete Protection.

Liquid dispensing

For accurate liquid dispensing, Flow Laboratories recommends its new line of lightweight **multi-channel pipettes** (*Reader Service No. 105*). The user simply dials in the required dispensing volume using a colour-coded thumb button. A digital display of the volume appears in an integral window, making it virtually impossible to make a mistake, says Flow.



Multichannel pipettes from Flow.

The pipettes come in three sizes for 4-, 8-, and 12-channel dispensing at a cost of £198 (UK), £247 (UK), and £308 (UK), respectively. Two colour-coded volume ranges are available: yellow for dispensing 5-50 µl in 0.5-µl increments and green for the 50-300 µl range in 5-µl increments. Pipette tips are supplied either sterile or nonsterile and can either be sold separately or in bands of four for rapid replacement during use. Flow can be found on stand C800.

Jencons Scientific Ltd, exhibiting on stand E178, has a **programmable liquid dispensing machine** — the Perimatic — which is suitable for aqueous solutions, sterile and shear-sensitive fluids (*Reader Service No. 106*). The machine uses a peristaltic pump to dispense volumes from 0.5 ml to 1,000 ml, and can dispense automatically at preselected intervals, or be operated manually or via a footswitch control. The Perimatic continuously recycles fluid even when it is not dispensing, which is useful for suspensions, says Jencons. Frothing and splashing can be prevented by programming the dispensing to begin and end slowly. Liquid is contained within the tubing at all times and a different dispensing head is used each time the solution is changed, eliminating

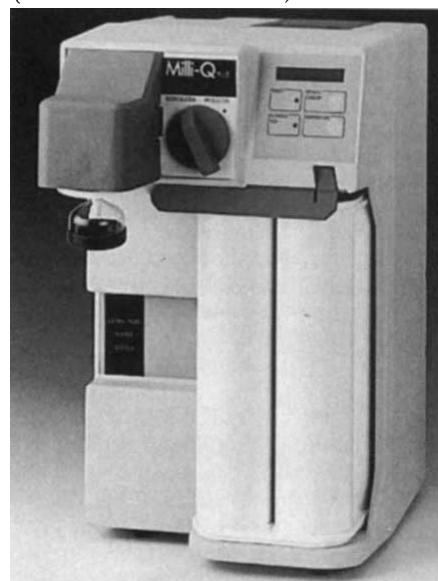


The Perimatic liquid dispenser from Jencons.

the possibility of contamination, says Jencons.

Pure and simple

Millipore Ltd will be featuring a new **water purification system** — the Milli-Q Plus — for general laboratory use or specialist applications such as HPLC, ion chromatography, TOC analysis, and atomic absorption spectrophotometry (*Reader Service No. 107*). The Milli-Q



The Milli-Q yields 1.5 litres of water per minute

Plus can provide 1.5 litres per minute of ultrapure water that is particle-free to 0.22 µm, has a 18 MΩ-cm resistivity, and less than 10 p.p.b. TOC if pretreated with reverse osmosis, says Millipore. The system uses Millipore's patented purification packs, which the company says are easy to remove and install. An alphanumeric display indicates the operational status of the system, and an alarm alerts the user of the need to change the purification packs. During periods of reduced demand, the system switches to a standby operation of intermittent recirculation. Prices for the Milli-Q Plus, which can be seen on stand E186, start at around £1,900 (UK).

If your research requires an apyrogenic water source, then Elga Ltd recommends its Elgastat UHP-UF **water purifier** (*Reader Service No. 108*). The unit uses a combination of organic adsorption, ion exchange, ultra-microfiltration, photo-oxidation and ultrafiltration technologies to provide ultra-pure water at flow rates of up to two litres per minute, says Elga. The £2,450 (UK) UHP-UF incorporates a 20,000 molecular weight cut-off ultrafilter to produce water that is particle-, pyrogen- and bacteria-free. Special features include a programmable rinse system that prevents organic contaminant build-up, and continuous recirculation which inhibits microbial growth and attachment. Each unit is fitted with a water-quality meter and an alarm which indicates when purity falls below the desired level. The UHP-



Elga's water purifier screens out pyrogens.

UF can be fed from raw mains, Elgastat Prima (reverse osmosis) or deionised water. Elga will be on stand E198.

The right environment

Autogene is the new compact **programmable waterbath** from Grant Instruments Ltd, who are exhibiting on stand H0150 (*Reader Service No. 109*). Designed for efficient thermal transfer and rapid automated temperature cycling from 0 to 99 °C, the waterbath offers considerable

advantages over metal thermostats, says the company. Autogene comes with removable test tube racks for a range of test tube sizes and a microtitre plate holder. The instrument can store up to nine run programs: an additional program is available for testing experimental protocols. Each program can include pre- and post-treatment parameters and can have up to 99 cycle repeats. Cycles can contain up to three setpoint/hold segments and the instrument provides a rapid rate of temperature change between setpoints, says Grant.

To overcome the problems associated with using water or oil for heat transfer, Jaytee Biosciences Ltd have come up with the BioOven — a **programmable oven** which uses forced air as a heating and cooling system (*Reader Service No. 110*). The £2,250 (UK) BioOven has a 500-cubic inch capacity and can hold 200 microcentrifuge tubes or four 96-well microtitre plates. Various heating and cooling modes are available, including isothermal operation and temperature cycling from ambient to 150 °C. Temperature sensors ensure a temperature variance of less than 1 °C, says Jaytee. The BioOven can store up to eight programs, each containing a maximum of six ramp/soak segments per program. The company says that the BioOven offers fast ramp rates of up to 1 °C per second. For added flexibility, programs can be linked together and can be set to operate 1-9999 cycle repeats. The BioOven can be seen on stand D822.

Visitors to stand A432, will be able to see Cleansphere — Safetech Ltd's futuristic-looking benchtop **sterile chamber** which has applications in fields as diverse as biotechnology, horticulture, microelectronics and pharmaceutical research (*Reader Service No. 111*). Cleansphere — developed in conjunction with University College, Cork — incorporates a Hepa filter cartridge and provides air-class 100 sterility, says Safetech. A built-in alarm



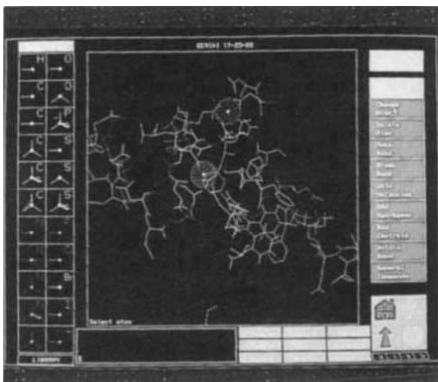
Safetech's Cleansphere sterile chamber.

indicates the sterility level and the need for filter replacement. The £830 (UK) unit provides all-round vision, a worksurface that is 400 mm in diameter, and easy hands-on access inside the chamber through hand bays. The unit can easily be dismantled for cleaning or access by removing the top-half of the chamber. A light source is fitted as standard and the unit can be treated against static.

IncuLAB is the portable **laboratory-based incubator** that Robin Instruments Ltd says is ideal for microbial analysis of faecal and total coliform (*Reader Service No. 112*). The instrument can be battery- or mains-operated and features a double incubator, each side of which can be set to run at a different temperature from 30 °C to 50 °C. The temperature variability of the incubator is ± 0.25 °C, says the company. IncuLAB can hold 50 aluminium petri dishes (25 per chamber), which can be autoclaved after use. The complete package includes the incubator and carrying case, two petri dish racks, filter unit with sample cup, lowering cable and vacuum pump, 50 petri dishes and a set of tweezers.

Clever computing

GEMINI is the **3-D real-time molecular modelling** and drug design software package that Hampden Data Services Ltd will be exhibiting, on stand B484 (*Reader*

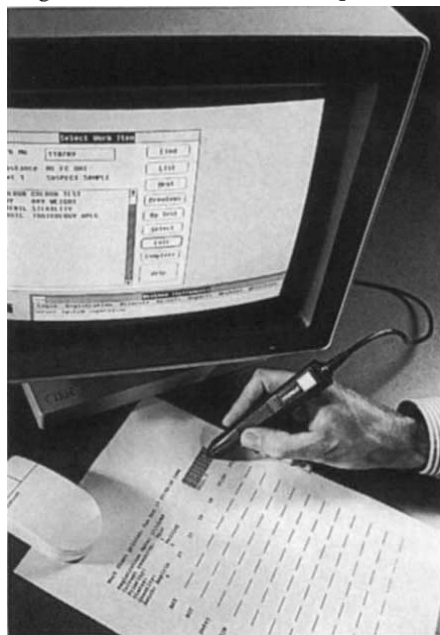


GEMINI molecular modelling software.

Service No. 113). Developed by the Institute of Cancer Research, UK, GEMINI provides a useful research tool for molecular design and analysis. Designed to run on Silicon Graphics' Personal Iris graphics work stations, the software offers a range of display modes, including solid and dotted surface, wireframe and electrostatic field representations. GEMINI's 'docking' facility allows the user to visualize the 'fit' of molecules. The 3-D images can be colour-coded, rotated and zoomed for detailed analysis, stored, incorporated into further structures, or used as 'building blocks' for more complex molecules. Other features include a cut-and-paste facility for on-screen editing, fragment library, and full compatibility with

chemical databases or quantum chemical and mechanics programs.

Beckman Instruments Ltd have teamed up the data management capability of large LIMS with the working ease of



Beckman's EasyLIMS uses Microsoft windows.

Microsoft windows to produce EasyLIMS — a **PC-based LIMS software package** designed to suit growing laboratory needs (*Reader Service No. 114*). Developed in collaboration with Harley Systems, the QuickStart turnkey version comes complete with IBM hardware, preloaded Microsoft and Beckman software, user manuals and mouse. Use of Microsoft windows brings data management and report generation within the reach of even inexperienced users, says Beckman. EasyLIMS has an integrated word processor and calculation facility, and post-analysis capabilities include integrated SQL database, spreadsheet, statistical analysis, and 3-D graphics. EasyLIMS can be used in a stand-alone system or networked with up to ten users. Beckman will be on stands G170/C524.

Showing for the first time on stand E210 will be VG Data Systems' new **chromatography handling product Xchrom** (*Reader Service No. 115*). The software is based on the X-window standard and offers the



The XChrom system from VG Data Systems.

improved reporting facilities associated with a window-based product, says the company. The software is available on two computer platforms: VAX with the VMS operating system and HP 9000 series with the HP/UX operating system. One of the advantages of X-chrom, says VG Data Systems, is the ability to export chromatograms into DECwrite for integration into reports prepared by word processing. The VG chromatography server is still employed as the acquisition device, which the company says provides the facilities for upgrading and expansion for existing Multichrom and Minichrom users, with the added advantage of a database.

Lab paraphernalia

On view for the first time, on stand number H160, will be the Wifug Technic M — the latest **bench-top centrifuge** from Eltex of Sweden Ltd (*Reader Service No.*



A bench-top centrifuge from Eltex.

116). This compact centrifuge can be fitted with both angle and swing-out rotors for 15-, 50-, and 100-ml tubes. The Technic M features a stepless speed control, timer and brake, and is capable of a top speed of 7,000 r.p.m. (5,055 g) when loaded with four 100-ml tubes, says the company. Safety aspects of the Technic M include a failsafe lock, imbalance cutout and stationary rotor indicator. The basic centrifuge without rotors is £900 (UK).

The CP Instrument Co. says its new **hand-held thermometer** has many applications in fields as diverse as food research to industrial production (*Reader Service No. 117*). The thermometer can record temperatures with either a 0.1 °C or 1.0 °C resolution for both centigrade and



CP Instrument's hand-held thermometer.

Fahrenheit. The device can store peak and trough temperatures and can perform differential temperature readings with one probe, eliminating possible probe error says the company. Other features include the facility to accept J, K, T and E thermocouples, 'hold' the display reading on all functions, be field-calibrated against known temperatures, and storage up to eight readings. At £149.50 (UK), the thermometer comes complete with plastic casing and a sealed membrane keypad. CP Instruments will be exhibiting a range of handy laboratory accessories on stand L1290.

Technique Ltd will be introducing Flo-Vac at British Lab Week — the multi-purpose **sample preparation unit** for digesting samples prior to chemical analysis or



Technique's Flo-Vac for sample digestion.

synthesis (*Reader Service No. 118*). Flo-Vac comes in two models: Flo-Vac 3 with an operating temperature range of 25 °C to 105 °C and Flo-Vac 3H with a range of 90 °C to 180 °C. Using the bench-top sample preparation unit, it is possible to perform several tasks *in situ* without interrupting the process. Flo-Vac can provide a controlled oxygen-free atmosphere, which Technic says is useful for oxygen-free digestions of amino acids. Reagents can be added and samples taken at any time. The unit is fitted with nitrogen gas inlet tubes and so gassing can also be performed *in situ*: a vacuum attachment can be used to speed up the evaporation process. The 210 × 330 × 400-mm unit can accommodate up to twelve 16-, 19- or 28-mm tubes. Technic will be on stand K1208.

For measuring

Dynatech Laboratories Ltd has a Micro-lite ML 1000 **scanning luminescence system** designed to automate sample screening for 'flash' or 'enhanced' bioluminescence or chemiluminescence reactions (*Reader Service No. 119*). On-board dedicated software offers selectable plate scans, the addition of up to three reagents, and various modes of data presentation. When the system is used with Microlite Removawell Strips, Dynatech says a sensitivity in the femtomole

range can be achieved without signal 'cross-talk'. The system can be programmed to elevate and maintain the microstrip temperature above ambient. Data processing functions include graphic analysis and mean, standard deviation and coefficient of variation calculations. The \$12,000-\$14,300 (US) model 3 system can be supplied with automatic reagent dispensers and a dot-matrix printer.

The PU8710 is the latest **UV-visible spectrophotometer** in the PU8700 Series from Philips Scientific (*Reader Service No. 120*). The PU8710 costs less than £6,000 (UK) and is based on the optical specifications of the PU8700. It has a 2-nm bandwidth and will scan over a range of 190-900 nm. The instrument has been combined with an IBM PC or compatible and Philips' FALCON colour graphics software. This configuration allows the user, via "pop-up" menus, help menus and prompts, to perform data processing functions irrespective of ability. The graphics and data manipulation functions

ADVERTISEMENTS

for IBM-compatible personal computers

Software for Your Lab

CELLINE Keeps accurate records of cell lines, cultures, freezes and ampule storage locations in all LN₂ and mechanical freezers.

GEL METRIC Calculates DNA and protein sizes from gel mobilities. Use with or without a digitizer.

SMGWS 2 Draws stereoscopic 3-D space-filling macromolecules on a PS/2 computer!

BioScience
SOFTWARE (814)692-7204

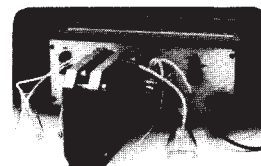
P.O. Box 10003, State College, PA 16805

Money-Back
Guarantee!

Reader Service No.26

LIPOSOMES

REPRODUCIBLE — HOMOGENEOUS —
EXTREMELY RAPID



Our new LIPOSOMAT guarantees rapid (5ml within 60 minutes) preparation of uniformly sized unilamellar liposomes. Liposome size is selectable between 25 and ca. 600 nm diameter. Sample volume is normally 5-10ml or up to 200ml if used in combination with an additional instrument. Lipid concentration can be up to 300 mg/ml.

DIANORM

POB 126, D-8000 Munich 65, FRG

Reader Service No.45



Philips's newest UV-visible spectrophotometer. include dual/triple display, zoom, spectral subtraction, multi-wavelength calculations, and quadratic and linear calibration curves. An optional plotter can provide hard copy in up to six colours. Philips Scientific will be exhibiting on stands D830 and E176.

Full system automation and scale-up facilities are features of the NovaPrep 5000 **semi-preparative HPLC system** from Technicol Ltd, which can be seen on stand K1210 (*Reader Service No. 121*). The system has a 10 to 50-mm column capability and can operate at pressures of up to 5,000 p.s.i., says Technicol. The interactive TurboPrep software package, designed to run on a 286/AT computer, allows full methods development capability and provides controlled gradient formation, sample injection and fraction collection. A special feature of the NovaPrep 5000 is the ContinuRinse pump design, which the company says can prolong the life of the pump's piston seals.

The QuikChem AE **automated ion analyser** features the combined power of flow injection analysis and ion chromatography in one instrument, and can be seen on the Roth Scientific Co. Ltd stand, number H140 (*reader service no. 122*). The system is capable of performing multichannel analysis with up to seven channels operating in parallel. IC can be performed either sequentially or simultaneously with FIA-based methods, with method switching taking less than 15 minutes, says Roth. The company says, QuikChem AE offers a sample throughput of 90-120 samples per hour, fast start-up and short analysis times, with sample processing taking less than one minute for

FIA. An integral part of the system is an IBM PC-XT compatible computer and QC/QA software, which provides automated system set-up, sampling, dilution. The system provides real-time data processing, including corrections for baseline drift, dilution and weight factor calculations, and interchannel relationship determination.

Biotech Instruments Ltd, exhibiting on stand L1254, are the UK distributors for Bioanalytical Systems' **BAS 200A chromatographic analyser** for PTH-amino acids, peptide mapping and free amino acids (*Reader Service No. 123*). The system uses a narrow-bore (0.21 cm) column for PTH-amino acid analysis, and has com-



puter-controlled start-up and operation. The company says, all 20 PTH derivatives can be resolved in less than 18 minutes, with cycle times of 30 minutes and a sensitivity of less than 200 femtomoles per compound. The BAS 200A can also be used for peptide mapping by virtue of the tandem variable wavelength UV and dual-channel amperometric detectors in the BAS 200A. The amperometric detector can also be used for the determination of free, primary amino acids using the OPA-tert butylthiol derivitization method developed by BAS, which the company says has a sensitivity of 50 femtomoles for gamma-aminobutyric acid.

Spectra FOCUS is the new real-time **scanning UV/Visible detector** for liquid chromatography that Spectra-Physics will be exhibiting, on stand E514 (*Reader Service No. 124*). The detector can be used for single- or multiple-wavelength operations. Easy-to-change flow cells for analytical, micro, biotech, and preparative LC or capillary electrophoresis, gives



The Spectra FOCUS scanning UV-visible detector.

the machine added flexibility, says the company. Dedicated chromatography software for use on an IBM PS/2 computer offers complete instrument control.

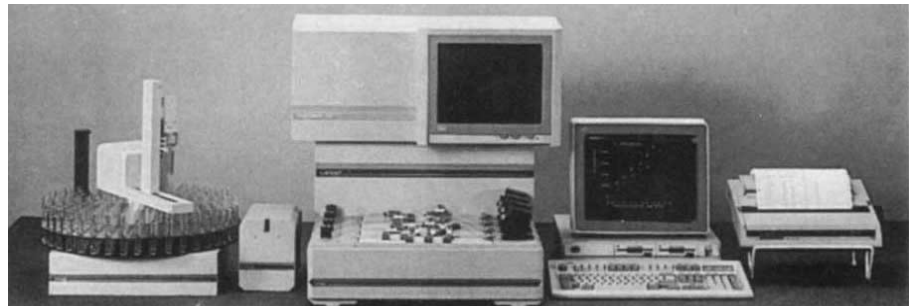
Budding anthropologists may be interested in the novel line of T-shirts, sweat shirts and ties depicting skulls of *Australopithecus boisei*, *Homo habilis* and *Homo erectus* (*Reader Service No. 125*). These fashion items were designed by Richard Leakey, palaeontologist and Director of the National Museum of Kenya, Nairobi, who licensed Schreter Neckwear of Baltimore to manufacture the products. The company's President, Harvey Schreter, has had a lifelong interest in anthropology and primitive art and hopes that the designs will be enjoyed by "genuine as well as arm-chair adventurers" alike. Harvey and Phillis Schreter witnessed Leakey's latest find first hand when, in 1988, they joined him on a dig in Lake Turkana, Northern Kenya, at the site where the Leakey team discovered the oldest complete prehistoric skeleton ever found — that of a 1,600,000 year-old *Homo erectus* boy. A proportion of the profits from the sale of these items



T-Shirts featuring ancient skulls.

will go towards funding the National Museum of Kenya and the ongoing research efforts of Leakey in East Africa. T-shirts sell for £12 (US), sweatshirts for \$25 (US), silk-blend ties for \$16.50 (US) and all-silk ties for \$25 (US).

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.



The QuikChem AB automated ion analyser from Roth Scientific.